

DOPAMINERGIC MODULATION OF OREXIN AND
MCH NEURONS (IN THE LATERAL HYPOTHALAMIC
AND PERIFORNICAL AREA)

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DOPAMINERGIC MODULATION OF OREXIN AND MCH NEURONS
(IN THE LATERAL HYPOTHALAMIC AND PERIFORNICAL AREA)

By

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Table of Abbreviations

| | |
|---------|---|
| ACSF | Artificial Cerebrospinal fluid |
| AgRP | Agouti-related protein |
| AMCA | Aminomethylcoumarin acetate |
| ArcN | Arcuate nucleus |
| CART | Cocaine- and amphetamine-regulated transcript |
| CCK | Cholesystokinin |
| CRF | Corticotropin-releasing factor |
| D1-like | D1/D5 dopamine receptors |
| D2-like | D2/D3/D4 dopamine receptors |
| DA | Dopamine |
| DNQX | 6,7-Dinitroquinoxaline-2,3-dione |
| EPSC | Excitatory postsynaptic current |
| EPSCs | Excitatory postsynaptic currents |
| GABA | γ -aminobutiric acid |
| ICSS | Intracranial self-stimulation |
| LH | Lateral hypothalamus |
| LH/PFA | Lateral hypothalamus/perifornical area |
| MCH | Melanin-concentrating hormone |
| mEPSCs | Miniature excitatory postsynaptic current |

| | |
|---------------|--|
| NAcc | Nucleus accumbens |
| NPY | Neuropeptide Y |
| PFC | Prefrontal Cortex |
| RMP | Resting membrane potential |
| SD | Sprague Dawley |
| TTX | Tetrodotoxin |
| VMH | Ventromedial hypothalamus |
| VTA | Ventral tegmental area |
| α -MSH | α -melanocyte stimulating hormone |

1 INTRODUCTION

Since animals cannot generate energy on their own, energy intake via food consumption is an absolute necessity. The irregularity of food availability in the wild has forced evolutionary adaptation to store and prepare for long-term energy requirements and to override short-term energy homeostasis. However, the environment for humans and companion animals has changed within the past half century, in which food is more readily available and the labor associated with food consumption (hunting, collecting, etc.) has greatly diminished. Evolution has not caught up with this drastic environmental change as our genes are still encouraging the intake of high-energy food. As was shown by a recent RAND study, obesity in the US is growing rapidly and morbid obesity in particular is rising three times faster (DeNoon, 2007). Obesity prevails as one of the top global health problems because it is known as a risk factor for various chronic disorders such as type II diabetes, cardiovascular disease, hypertension and certain forms of cancer. (World Health Organization, 2007). In order to control obesity, it is important to understand how energy homeostasis and appetite for food is controlled.

The motivation to eat palatable food is not only driven by its nutritional value, but also by the brain's ability to recognize energy dense food as a tasty and palatable stimulus. Behavioral responses to the reinforcing nature of palatable food involve a wide range of brain areas and neurochemicals, particularly those involved in reward and energy homeostasis (DiLeone et al., 2003; Erlanson-Albertsson, 2005; Saper et al., 2002). Among these, the lateral hypothalamus (LH) has been recognized as a common brain area

that mediates both feeding and reward-related behaviour (MARGULES and OLDS, 1962). Furthermore, recent studies suggest that orexin-expressing neurons may be mediating the feeding and reward-related functions of the LH (Hirasawa et al., 2007). Therefore, investigating the physiology and pathology of orexin neurons may help us to understand the central mechanism of energy homeostasis and food reward. This thesis focuses on the interaction of dopamine (DA) and orexins neurons, two key players in reward and energy homeostasis.

1.1 Hypothalamic control of energy homeostasis

An important site for central regulation of food intake is the hypothalamus. Brain lesion and stimulation studies first implicated the LH as the “hunger center” and the ventromedial hypothalamus (VMH) as the “satiety center” (Stellar, 1954). Although we now know that this ‘dual center’ model is oversimplified (Schwartz et al., 2000), these early studies indicated that the hypothalamus plays a critical role in food intake. More recent research suggests that there is a hypothalamic neural network that plays a central role in energy homeostasis. This network includes the arcuate nucleus (ArcN), LH, VMH, paraventricular nucleus and dorsomedial hypothalamus (Schwartz et al., 2000). The ArcN is located adjacent to the median eminence that has a fenestrated blood brain barrier through which nutrient signals from the circulation are believed to enter and diffuse into the ArcN (Schwartz et al., 2000; van Den Pol, 2003). This allows neurons in the ArcN to work as sensors for the levels of circulating nutrient signals, including

glucose, leptin, ghrelin and insulin (van Den Pol, 2003). ArcN neurons in turn project to other hypothalamic nuclei to form the neural network for energy homeostasis (Saper et al., 2002; Schwartz et al., 2000; Spiegelman and Flier, 2001; Inui, 2000a).

Within this neural network, multiple neuropeptides have been identified as modulators of food intake. Neuropeptides that inhibit feeding (anorexigenic peptides) include α -melanocyte stimulating hormone (α -MSH), cocaine- and amphetamine-regulated transcript (CART), corticotropin-releasing factor (CRF), cholecystokinin (CCK) and neurotensin (Schwartz et al., 2000). Appetite-inducing (orexigenic) peptides include neuropeptide Y (NPY), agouti-related protein (AgRP), galanin, orexin, melanin-concentrating hormone (MCH) and ghrelin (Nakazato et al., 2001; Schwartz et al., 2000).

The ArcN contains at least two populations of neurons that produce peptides with opposite action on food intake. One population of ArcN neurons produces NPY and AgRP. Intracerebroventricular administered show a rapid and robust feeding effect after a bolus of NPY application (Edwards et al., 1999; Kalra et al., 1999). Also NPY neurons show increase in NPY mRNA expression in response to insulin treatment (Wilding et al., 1993; Williams et al., 1989). Central administration of AgRP also produces a significant increase in food intake at 24 hrs post injection (Rossi et al., 1998). It is known that AgRP is a potent and selective endogenous antagonist for MC3/MC4 melanocortin receptors (Yang et al., 1999b; Yang et al., 1999a). Another population of ArcN neurons produces α -MSH which derives from the precursor pro-opiomelanocortin (POMC) (Chen et al., 1999; Jobst et al., 2004) and binds to MC3/MC4 receptors, reducing food intake and body weight (van Den Pol, 2003). Both NPY/AgRP and POMC neurons are known to

innervate the LH as well as other hypothalamic nuclei involved in energy homeostasis (Elias et al., 1998; Inui, 2000b).

Two neuropeptides highly expressed by the LH and perifornical area (LH/PFA) are MCH (Bittencourt et al., 1992) and orexins (de Lecea et al., 1998; Sakurai et al., 1998) and are thought to provide the neuronal substrate for LH functions related to food intake. MCH and orexins are found in separate but spatially overlapping populations of neurons in animal and human brain (Broberger et al., 1998; Elias et al., 1998; Peyron et al., 1998). Both types of neurons receive direct projections from NPY/AgRP and POMC neurons of the ArcN (Elias *et al.*, 1998) and have similar wide-ranging projections to a number of structures including: cerebral cortex, hippocampus, hypothalamus, thalamus, brain stem and spinal cord (Bittencourt et al., 1992; Broberger et al., 1998; Chemelli et al., 1999; Peyron et al., 1998).

1.2 The LH as part of the reward circuit

While a negative energy balance is sensed by the hypothalamus and stimulates food intake, the amount of food eaten is also influenced by environmental factors. Among the environmental factors, the availability of palatable foods is the most obvious (Volkow and Wise, 2005). It is known that the rewarding nature of food activates brain circuits involved in reward, motivation and decision-making (Volkow et al., 2004). The experience or expectation of reward is associated with the activity of the mesolimbic dopamine system that originates from the midbrain ventral tegmental area (VTA) and

projects to forebrain structures including the nucleus accumbens (NAcc) (Berridge and Robinson, 1998; Kelley and Berridge, 2002).

In addition, the LH has been seen as an integral part of a larger reward circuit (DiLeone et al., 2003; Kelley et al., 2005). Stimulation studies demonstrated that rats will self-administer electrical stimulation (intracranial self-stimulation; ICSS) directly to the LH (OLDS and MILNER, 1954). This behaviour is dependent at least in part on direct stimulation of intrinsic neurons of the LH because chemical lesions selectively destroying cell bodies in the LH attenuate ICSS response (Velley et al., 1983). It was recognized early that the LH is a common brain area that mediates both feeding and reward-related behaviour. Electrode placements in the LH that induced food intake were also sites which generated high rates of self-stimulation (MARGULES and OLDS, 1962). Recent studies suggest that neuropeptides expressed in the LH play a critical role in food intake and reinforcement (DiLeone et al., 2003).

The LH also receives inputs from the amygdala and integrates the environmental and emotional signals that are associated with food delivery (Kirouac and Ganguly, 1995; Petrovich et al., 2002; Petrovich et al., 2005), which can override satiety (Petrovich et al., 2002). Thus, the LH not only mediates phagic functions (Saper, 1985; Saper et al., 1986) but also cognitive, reward- and emotion-related functions (Berthoud, 2002; Saper et al., 2002).

1.3 Orexin neurons

One of the primary cellular substrates that mediate the function of the LH may be orexin neurons. Majority of orexin expression is concentrated in the LH. Although, Pre-pro orexin mRNA also has been detected in the testis (Sakurai et al., 1998 ;Johren et al., 2001) stomach, lung and kidney (Yan et al., 2005). Orexins (orexin A and B, also known as hypocretin 1 and 2) were originally identified as orexigenic neuropeptides, because they induce feeding when injected into the cerebral ventricles (Edwards et al., 1999; Haynes et al., 1999; Sakurai et al., 1998; Yamanaka et al., 1999) or the LH (Sweet et al., 1999) and orexin receptor antagonists conversely decreases food intake (Haynes et al., 2002). Orexin neurons are sensitive to nutritional states such as fasting and hypoglycemia where they show changes in c-Fos expression (Cai et al., 2001; Moriguchi et al., 1999; Sakurai et al., 1998; Tritos et al., 2001; Yamamoto et al., 2000). However, more recent studies have provided evidence that orexin is not just an orexigenic factor.

It is now thought that orexins are responsible for the motivational and hedonic aspects of feeding. Orexins have been shown to mediate behavioural responses to addictive drugs (Borgland et al., 2006; Boutrel et al., 2005; Georgescu et al., 2003; Harris et al., 2005; Narita et al., 2006) and facilitate responses to natural rewards such as palatable food (Clegg et al., 2002; Furudono et al., 2006; Thorpe et al., 2005). Conversely, intake of palatable food stimulates orexin neurons. For example, high-fat diet and calorie-free sweetener have been shown to elevate orexin gene expression (Furudono et al., 2006; Park et al., 2004; Wortley et al., 2003). Thus, the intake of pleasurable food is associated with increased orexin neuron activity which acts in turn to promote further

intake of such food.

Furthermore, orexins play a critical role in stabilizing arousal (Mochizuki et al., 2004; Saper et al., 2005) and lack of orexin signalling leads to narcolepsy (Chemelli et al., 1999; Hara et al., 2001; Lin et al., 1999). Also, orexins have been shown to induce spontaneous physical activity (Kotz, 2006; Nakamura et al., 2000; Sunter et al., 2001; Thorpe and Kotz, 2005), increase sympathetic outflow (Ferguson and Samson, 2003; Follwell and Ferguson, 2002; Samson et al., 2002; Samson et al., 2005; Smith et al., 2002) and thermogenesis (Oldfield et al., 2002; Yasuda et al., 2005; Yoshimichi et al., 2001).

Collectively, orexin neurons seem to coordinate arousal, sympathetic and physical activity as well as the motivation to seek/eat food. Proper coordination of these functions is vital for survival because there will be a greater chance of finding and consuming food while staying vigilant to potential threats. This property of orexin neurons is manifested as food anticipatory behaviour or fasting-induced arousal that disappears in mice with genetic ablation of these neurons (Akiyama et al., 2004; Yamanaka et al., 2003).

Orexin A and B are 33- and 28-amino acid peptides, respectively (de Lecea et al., 1998; Sakurai et al., 1998) and are coded by the same gene located on chromosome 17q21 (Sakurai et al., 1998). There are two subtypes of orexin receptors, namely orexin-1 and orexin-2 receptors that have extensive yet distinct expression patterns in the brain (Marcus et al., 2001; Trivedi et al., 1998). Orexin-1 receptor shows higher affinity for orexin A, while orexin-2 receptor shows equal affinity for both orexin A and B (Ammoun et al., 2003; Sakurai et al., 1998). Both subtypes are G-protein coupled; orexin-1 receptor

seems to signal through Gq while orexin-2 couple to Gi/Go and Gq subunits (Sakurai et al., 1998; van Den Pol et al., 1998).

1.4 MCH neurons

Another population of neurons that are expressed exclusively in the LH and that may mediate the known LH function on food intake and reward is MCH neurons (Nahon, 1994). MCH induces feeding and suppresses energy expenditure when overexpressed or injected centrally (Ito et al., 2003; Ludwig et al., 2001; Nahon, 1994; Qu et al., 1996; Rossi et al., 1999; Shimada et al., 1998). On the contrary, MCH knockout mice are hypophagic and lean (Shimada et al., 1998). MCH is also known to mediate responses to rewarding stimuli such as food and alcohol (Duncan et al., 2005; Georgescu et al., 2005).

In addition, it has been shown that blockade or genetic deletion of MCH receptor subtype 1 (MCH1R; the cognate MCH receptors in rodents) results in antidepressant-like and anxiolytic phenotype in animals (Borowsky et al., 2002; Chaki et al., 2005; Georgescu et al., 2005; Roy et al., 2006b; Roy et al., 2006a; Takekawa et al., 2002). MCH also activates the hypothalamo-pituitary-adrenal axis (Kennedy et al., 2003; Smith et al., 2005) Thus, MCH has implications in motivation, mood and stress response which in turn may influence food intake and response to reward.

MCH was discovered in fish pituitary as a hormone that mediated color changes in the skin of the teleost fish (Kawauchi et al., 1983). After several years, MCH was described in mammals as a 19 amino acid cyclic neuropeptide which is identical in all

mammals including the mouse, rat, rabbit and human [reviewed in (Pissios and Maratos-Flier, 2003)]. MCH neuropeptide is expressed in the LH and zona incerta (Bittencourt et al., 1992). Also, MCH mRNA and pro MCH-derived peptide were founded in genitals and gastrointestinal tracts of rats (Hervieu and Nahon, 1995). In contrast to fish, MCH has no association to mammalian pigmentation (Pissios and Maratos-Flier, 2003).

MCH binds to two kind of receptors in the brain, MCH-1 and MCH-2 receptors, which are G protein-coupled that activate Gi, Go or Gq subunits (Hawes et al., 2000; Maulon-Feraille et al., 2002). Interestingly, MCH-2 receptors are not present in all species. Rodents apparently do not have MCH-2 receptors. Pigs and rabbits have non-functional alleles, whereas dogs, monkeys and humans have both MCH-1 and MCH-2 receptors (Tan et al., 2002). These receptors are expressed in various brain areas implicated in energy balance, as well as in cell groups involved in other aspects of feeding behaviour such as olfaction, motivation/reward, mastication and swallowing (Saito et al., 2001). The activation of these receptors modulates intracellular calcium concentration (Bachner et al., 1999; Chambers et al., 1999; Gao and van Den Pol, 2001; Gao and van Den Pol, 2002; Hawes et al., 2000; Lembo et al., 1999) or synaptic transmission (Gao and van Den Pol, 2001; Gao and van Den Pol, 2002; Varas et al., 2002).

1.5 Role of dopamine in food intake and motivation

Dopamine (DA) is another critical player in food intake and reward. Genotypes that alter DA reuptake are known to have a strong influence on food reinforcement and

weight gain (Epstein and LeDdy, 2006). DA release in the NAcc is associated with palatable food intake (Hajnal and Norgren, 2001; Masi et al., 2001), and motivation to eat requires this DA system to be intact. Animals with inactivated tyrosine hydroxylase in DA neurons will not eat enough to survive even though they can seek and ingest food (Szczypka et al., 1999).

On the contrary, genetic backgrounds that manifest lower DA signaling (higher DA reuptake or lower receptor expression) are associated with a stronger response to food reward and obesity in humans and animals (Epstein et al., 2004; Epstein and LeDdy, 2006; Figlewicz et al., 1998). Conversely, blocking DA reuptake, which increases the synaptic DA level in the brain, causes anorexia and weight loss in obese subjects (Epstein et al., 2004). It has been postulated that less DA signaling in the brain means reduced sensitivity of the mesolimbic system to reward, causing the subject to consume more food to compensate for the reduced sensitivity (Wang et al., 2001). While this is possible, it may also be due to inhibition of DA action in the hypothalamus.

Available evidence suggests that DA's inhibitory influence on food intake involves the hypothalamus. In the LH/PFA, DA receptor activation suppresses feeding (Leibowitz, 1975; Leibowitz and Rossakis, 1979; Leibowitz et al., 1986; Parada et al., 1988; Yang et al., 1997), whereas the D2-like receptor antagonist blocks the anorexic effect of DA (Leibowitz, 1975; Leibowitz and Rossakis, 1978; Parada et al., 1988). Endogenous DA release in this area is associated with food intake, suggesting it may be a satiety signal (Meguid et al., 1995; Yang and Meguid, 1995). DA is known to increase latency to meal onset and reduce meal size and duration (Fetissov et al., 2000; Leibowitz

et al., 1986). Also DA receptor agonists normalize hyperphagia and rectify metabolic/endocrine abnormalities, resulting in an improvement of obese-diabetic syndrome in leptin deficient ob/ob mice (Cincotta et al., 1997; Scisłowski et al., 1999). Furthermore, DA receptor expression and DA release in the LH/PFA are modulated by different energy states such as fasting, obesity and anorexia (Fetissov et al., 2000; Fetissov et al., 2002; Sato et al., 2001).

DA action within the LH/PFA is also known to inhibit the reward circuitry, the mesolimbic DA system. Injection of the D2-like receptor antagonist sulpiride into the LH/PFA results in DA release in the NAcc (Morutto and Phillips, 1998b; Parada et al., 1995) and induces robust locomotor activity and conditioned place preference through this mechanism (Morutto and Phillips, 1998a). In fact, rats will self-inject sulpiride into the LH/PFA consistent with it being a rewarding stimulus (Parada et al., 1995). Given that the bulk of DA fibers in the LH/PFA projects from the VTA (Leibowitz and Brown, 1980; Yoshida et al., 2006), the inhibitory DA effect in the hypothalamus may be a feedback mechanism for fine-tuning the excitatory hypothalamic influence to the mesolimbic DA system.

1.6 Effect of glutamate and GABA on food intake

It has been suggested that glutamate and γ -aminobutyric acid (GABA) regulates hypothalamic control of food intake (Maldonado-Irizarry et al., 1995; Stanley et al., 1996; Tsujii and Bray, 1991; van Den Pol, 2003). Endogenous glutamate release occurs during meal initiation and GABA release during satiation in the LH/PFA where orexin

and MCH neurons exist (Rada et al., 2003). In the same area, injection of glutamate or its agonists receptors elicits intense feeding (Duva et al., 2005; Stanley et al., 1993) while infusion of picrotoxin, a non-competitive GABAA-antagonist increased food intake (Tsujii and Bray, 1991). Therefore, glutamate and GABA play a critical role in the control of food intake by the LH.

It has been shown that orexin and MCH neurons receive synaptic inputs mediated by glutamate and GABA, respectively. Glutamate mediates excitatory synaptic transmission, whereas GABA mediates the inhibitory synaptic inputs. Modulation of these synaptic inputs is one mechanism by which the firing activity of the postsynaptic orexin and MCH neuron can be altered (Acuna-Goycolea et al., 2004; Guyon et al., 2005; Huang et al., 2007; Li et al., 2002).

2 Aim

Based on the current literature, it is possible that DA release in the LH/PFA modulates energy states of the animal, like obesity or anorexia, through its action on orexin and MCH neurons.

The goals of this thesis were two fold. First, we sought to determine a standard to identify orexin and MCH neurons using their electrophysiological characteristics. Because these neurons are found to be spatially overlapping in the same hypothalamic area, establishing such criteria would enhance our ability to perform studies on identified neurons. Second, we tested the effect of DA on synaptic transmission to orexins and MCH neurons, to explore the cellular mechanism of DA action in the LH/PFA.

3 Materials and Methods

3.1 Animal model

Sprague Dawley (SD) rats were used in this study. Male SD rats between 60 to 100 grams were obtained from Memorial University Vivarium. The rats were housed with food and water available *ad-libitum* and with controlled room temperature (21°C, $\pm 2^\circ\text{C}$), humidity, air renovation and light/dark cycle controlled (12/12hrs). All experiments were performed in accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the Memorial University Institutional Animal Care Committee. Attention was paid to use the minimum number of animals necessary to ensure results were consistent. Nonetheless, in each set of experiments, no more than two cells were used that derived from an individual animal.

3.2 Slice preparation

Rats were decapitated using a guillotine under deep halothane anesthesia. The brain was rapidly removed and placed for two minutes in ice-cold buffer solution (0–2°C) composed of the following (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4 , 7 MgCl_2 , 0.5 CaCl_2 , 25 NaHCO_3 , 25 glucose, 30 sucrose, 3 pyruvic acid, 1 ascorbic acid. A hypothalamic block was prepared and 250- μm -thick coronal slices were obtained on a

vibrating-blade microtome (Leica Microsystems, model VT1000S) in the same ice-cold buffer solution. Slices were incubated at 33–34°C for forty five minutes in order to allow neurons recover from the mechanical stress produced by the sectioning process. Then slices were kept at room temperature until recording. The samples were maintained in artificial cerebrospinal fluid (ACSF) before and during recordings. It was composed of the following (in mM): 126 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 10 glucose, 1 ascorbic acid. Both solutions were continuously bubbled with a gas mixture of O₂ (95%) and CO₂ (5%).

3.3 Electrophysiological recordings

A hemisected slice was transferred into a linear flow small volume perfusion recording chamber (Dagan Corporation, model RCP-6T) and was perfused at 1.5–2 ml/min with ACSF. The chamber temperature was set at 33–34°C with a temperature controller (Dagan Corporation, model HW-30). Whole-cell patch-clamp recording was performed using a Multiclamp 700B amplifier (Axon Instruments, Inc.) and pClamp 9.2 software (Axon Instruments, Inc.). With the visual guidance by infrared-differential interference contrast (IR-DIC) microscope (Leica Microsystems, model DM LFSA), neurons adjacent to the fornix with a diameter between 10 to 20 µm were selected. The recording electrode was made of borosilicate glass (Garner Glass Company) using a Sutter micropipette puller (Sutter Instrument Co., model P-97). The resistance of the electrode was 4-7 MΩ when filled with the internal recording solution containing the

following (in mM): 123 K-gluconate, 2 MgCl_2 , 8 KCl, 0.2 EGTA, 10 HEPES, 4 Na_2 -ATP, 0.3 Na-GTP, pH 7.3. The electrode was advanced to the cell until the tip touched the membrane. After a $\text{G}\Omega$ seal was made, negative pressure was applied to break the membrane and obtain the whole-cell configuration. Membrane potentials and currents were filtered at 1 kHz, digitized at 5 kHz and stored for off-line analysis.

3.4 Synaptic currents

As was described earlier by Fatt and Katz in 1952 spontaneous subthreshold electrical activity, called miniature events can be recorded from postsynaptic cells with an intracellular electrode. Synaptic currents can be monitored using Voltage-clamp technique and spontaneous synaptic currents, like action potential-independent miniature events, can be recorded in the presence of TTX. These miniature events are the result of quantal neurotransmitter release from the presynaptic terminal to the synaptic cleft in a random manner (FATT and KATZ, 1952; DEL CASTILLO and KATZ, 1954). Quantal can be defined as a minimum unit of neurotransmitter contained in a single vesicle in the presynaptic terminal that produce a visible postsynaptic response (DEL CASTILLO and KATZ, 1954). Voltage-clamp experiments were performed at a holding potential of -80 mV, to facilitate the driving force of ions passing through AMPA/Kainate receptors. With this condition spontaneous miniature postsynaptic currents were continuously monitored. A 20 mV-hyperpolarizing pulse lasting for 100 ms was applied every 20-60 s throughout each experiment, and the steady-state current and decay rate of the capacitance transient

were monitored as measures of input resistance and series/access resistance, respectively. Cells that showed significant change in these parameters were excluded from additional analysis, because such changes in recording condition can influence the results.

Miniatures events were pharmacologically isolated with tetrodotoxin (TTX) and picrotoxin application. After the effect of these compound stabilized (7 minutes), miniatures EPSCs (mEPSCs) frequency and amplitude were analyzed and the average of three consecutive minutes immediately prior to drug application was used as control to normalized data. For time effect plots, mEPSCs frequency and amplitude were analyzed and the mean values were calculated for each minute. For drugs tests, a period of three minutes following five minutes of drug application was considered as drug effects. This criterion was used throughout this study.

3.4.1 Electrophysiological characterization of LH/PFA neurons

In current clamp mode, the cells were injected with a series of five 200 ms-step pulses ranging from -300 pA to +200 pA in 100 pA increments. Every cell fired action potentials in response to depolarizing current steps. When the time interval between the last two spikes was longer by 30% or more in comparison with the two first spikes, we called it spike adaptation.

3.4.2 Action potential recording

The current-clamp mode was used to record spontaneous firing activity, without any current injection.

3.5 Data analysis

Miniatures EPSCs and action potentials were detected using Mini Analysis 6.0 software (Synptosoft, Inc.). The data are expressed as mean \pm SEM. Statistical comparisons were performed by using appropriate statistics i.e., Kolmogorov-Smirnov test for testing individual cells. Unpaired or paired Student t-tests for group comparison. A value of $p < 0.05$ was considered significant.

3.6 Immunohistochemistry

For immunohistochemical identification of the recorded cell, biocytin (1-1.5 mg/ml) was included in the internal solution. Immediately after the recording, slices were placed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C, then washed and stored in PBS. Slices were then washed in PBS, followed by incubation with anti-orexin A goat polyclonal IgG (1:3000 dilution) and anti-melanin-concentrating hormone (MCH) rabbit polyclonal IgG (1:2000 dilution) for three days at 4°C. Slices were then washed and treated for three hours with a combination of indocarbocyanine (Cy3) conjugated donkey

anti-goat antibody, cyanine (Cy2) conjugated donkey anti-rabbit antibody, and streptavidin-conjugated aminomethylcoumarin acetate (AMCA), all at 1:500 dilutions at room temperature. Antibodies were diluted with PBS with 0.05% Triton X. Slices were then washed, mounted, and examined under a conventional fluorescence microscope (Olympus, BX 51) for detection of orexin A (Cy3), MCH (Cy2) immunoreactivity, and biocytin (AMCA). 40 X images of Cy2, Cy3 and AMCA fluorescence were captured individually. Contrast of the images was improved and amalgamated using Adobe Photoshop 5.5 (Adobe Systems Inc.).

3.7 Chemical compounds

All drugs were bath perfused at final concentrations as indicated, by diluting aliquots of 1000 x stock in the ACSF immediately before use. Dopamine (DA) stock and the solutions included ascorbic acid (1 mM) and were light protected during storage and recordings to minimize oxidation. The final concentration of DMSO used as a vehicle was 0.1 %. SKF 81297, quinpirole, SCH 23390 and sulpiride were purchased from Tocris Bioscience (Ellisville, MO), DA, biocytin, picrotoxin from Sigma-Aldrich (St. Louis, MO), tetrodotoxin (TTX) from Alomone Labs (Jerusalem, Israel). Anti-orexin A goat polyclonal IgG was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-melanin-concentrating hormone (MCH) rabbit polyclonal IgG was from Phoenix Pharmaceuticals (Belmont, CA).

4 Results

4.1 Identification of neurons in the LH

In hypothalamic slices, sixty-three neurons were filled with biocytin and the phenotype of each neuron was confirmed using the immunohistochemical technique.

These sixty-three cells were divided in three different types according to their electrophysiological characteristics (Fig. 1, 2, 3 and 5). In Type 1 we included fifty-two neurons that showed a depolarizing sag (I_h current) in response to hyperpolarizing pulses from the resting membrane potential (RMP) (98.1 %, 51 of 52) and a rebound depolarization at the current offset (98.1 %, 51 of 52). The majority of them displayed no spike adaptation (69.1 %, 36 of 52), while others showed a different degree of spike adaptation. Also these neurons presented spontaneous firing at rest (90.4 %, 47 of 52). The RMP for these fifty-two cells were between -39.0 to -62.0 mV (Fig. 1). RMP was measured as the stable membrane potential recorded in between synaptic potentials without any current injection.

Among these fifty-two cells, two of them presented a RMP of -60.0 mV or lower (Fig. 1B) and were immunonegative for both orexin A and MCH. There were no criteria that could be used to further distinguish different phenotypes in the remaining group. Of these remaining fifty neurons, three were non-orexin, non-MCH neurons and forty-seven of them were orexin A-immunopositive neurons (Fig. 4A). This result suggests that the majority of the Type 1 cells that present a RMP above -60mV are orexin neurons (94%,

47 of 50). The average RMP for neurons that were orexin A immunopositive was -48.6 ± 0.8 mV.

In contrast, nine neurons were grouped as Type 2 (Fig. 2) that showed no Ih current (100 %, 9 of 9) nor rebound depolarization (100 %, 9 of 9) in response to a hyperpolarizing pulse from the RMP. The average RMP for these neurons was -58.8 ± 1.9 mV and a majority of them did not fire spontaneously (88.9 %, 8 of 9). Furthermore, eight of nine cells showed a clear spike adaptation (88.9 %; Fig. 2). All of these neurons were immunopositive for MCH (Fig. 4B).

Finally, Type 3 included two neurons (Fig. 3). These cells presented no Ih current and fired an action potential when released from a hyperpolarizing current step. The RMP was -50.5 ± 9.5 mV and there was no spontaneous firing at rest. In response to positive current injection, they initially fired an action potential but ceased further activity (Fig. 3). These neurons were non-orexin, non-MCH neurons.

According to the results shown above, orexin and MCH neurons in the LH/PFA can be identified by their distinct electrophysiological features (Fig. 1, 2 and 5). For the rest of the study, these characteristics were mainly used to distinguish the phenotype of recorded neurons.

4.2 Synaptic currents in orexin neurons

Spontaneous postsynaptic currents were recorded in orexin neurons. Addition of 50 μ M picrotoxin (non-competitive GABA-A antagonist) in the bath reduced the

frequency of spontaneous postsynaptic currents and spontaneous excitatory postsynaptic currents (EPSCs) were monitored in isolation (Fig. 6). The frequency of spontaneous EPSCs was 2.5 ± 0.7 Hz ($n=5$). Miniature EPSCs (mEPSCs) were recorded in the presence of picrotoxin $50 \mu\text{M}$ and TTX $1 \mu\text{M}$ which showed a frequency of 1.5 ± 0.2 Hz ($n=30$) and an amplitude of 14.7 ± 0.7 pA, where we were able to record good examples of miniature and measured the amplitude just in six cells to be compared with the control. The frequency of spontaneous EPSCs was significantly higher than the frequency of mEPSCs ($n=5$, $p<0.05$). Almost all mEPSCs were abolished by DNQX $10 \mu\text{M}$ application (the AMPA/kainite receptor antagonist) (Fig. 6; $n=3$). Thus, in orexin neurons, the excitatory synaptic transmission is mediated by glutamate.

4.3 Dopamine Effect on orexin neurons

To test if dopamine (DA) induced any change in the excitatory synaptic transmission on orexin neurons, various DA concentrations were applied for a period of five minutes. When $1 \mu\text{M}$ DA was applied (Fig. 7A-C), it induced a reversible and significant increase in the frequency of mEPSCs in orexin neurons from 2.9 ± 0.6 Hz to 3.8 ± 0.6 Hz ($n=9$; $p<0.05$). In contrast, a high DA concentration ($100 \mu\text{M}$; Fig. 8A-C) induced a significant decrease in mEPSCs frequency from 3.3 ± 0.7 Hz to 2.0 ± 0.5 Hz, which was reversible upon wash ($n=9$, $p<0.01$). Overall, as shown in Fig. 9, we found that the direction of the DA effect was concentration dependent: $1 \mu\text{M}$ increased, whereas 10 and $100 \mu\text{M}$ decreased mEPSCs frequency ($10 \mu\text{M}$, control condition 4.6 ± 1.2 Hz and

DA application 3.3 ± 0.9 Hz, $n=5$, $p<0.05$). DA $0.1 \mu\text{M}$ had no effect in four cells tested (control condition was 5.2 ± 1.6 Hz and 5.2 ± 1.4 Hz in DA application) ($p>0.05$; Fig. 9). There was no significant change in the amplitude of mEPSCs in every dose of DA tested, even when there was a significant change in the frequency of mEPSCs (Fig. 7D; 8D) ($1 \mu\text{M}$, $n=9$, $p>0.05$; 10 - $100 \mu\text{M}$, $n=9$, $p>0.05$), indicating that the locus of DA action is most likely presynaptic instead of postsynaptic.

4.4 Role of D1-like receptors

It is possible that the bidirectional effect of DA on mEPSCs results from an activation of different subtypes of DA receptors, such as D1-like (D1/D5 dopamine receptors) or D2-like (D2/D3/D4 dopamine receptors) receptors. To test whether D1-like receptors could mediate the synaptic effects of DA in orexin neurons, SKF 81297 $10 \mu\text{M}$ (the D1-like receptor agonist) was applied for a period of five minutes. SKF 81297 significantly increased the frequency of mEPSCs ($n=9$, $p<0.05$) without affecting the amplitude ($p>0.05$; Fig. 10A-D). The effect of SKF 81297 was not significantly different from that of DA $1 \mu\text{M}$ ($p>0.05$). Thus, we hypothesized that SKF 81297 and DA $1 \mu\text{M}$ act on a common target, i.e., D1-like receptor. To test this hypothesis, SCH 23390 $10 \mu\text{M}$ (the D1-like receptor antagonist) was bath applied for five minutes prior to and during DA $1 \mu\text{M}$ application (Fig. 11A). We found that SCH 23390 not only blocked DA $1 \mu\text{M}$ -induced facilitation of mEPSCs, but also unmasked an inhibitory effect of DA on mEPSCs ($n=5$, $p<0.05$; Fig. 11B). Therefore, these results are consistent with the

excitatory effect of DA 1 μ M being mediated by D1-like receptors.

4.5 Role of D2-like receptors

To test whether D2-like receptors also modulated the excitatory synapses to orexin neurons, D2-like receptor specific agonist and antagonist were examined. Quinpirole 10-50 μ M (the D2-like agonist) application for five minutes reduced the frequency of mEPSC ($n=6$, $p<0.05$) without altering the amplitude ($p>0.05$; Fig. 12A-D). This inhibitory effect was similar to that of 100 μ M DA ($p>0.05$), from which we conclude that high dose of DA may activate D2 receptors. Therefore, we tested the effect of the D2-like receptor antagonist sulpiride on DA 100 μ M-induced inhibitory effect. Sulpiride 10 μ M was applied five minutes prior to and during 100 μ M DA application (Fig. 13A). In this condition, DA did not alter the frequency of mEPSCs ($n=4$, $p>0.05$). This result is consistent with inhibitory effect of a high concentration of DA being mediated by D2-like receptors (Fig. 13B).

4.6 Effect of Dopamine on Spontaneous Firing Activity

Next we tested whether the effects of DA on excitatory transmission could alter the firing activity of orexin neurons. In current-clamp mode, spontaneous action potentials were monitored in the presence of picrotoxin to block the influence of inhibitory synaptic inputs. The recorded orexin neurons showed spontaneous firing with

an average frequency of 0.7 ± 0.2 Hz ($n=5$). In this condition, three minutes of $1 \mu\text{M}$ DA application significantly increased the frequency of firing by 201.9 % of control, which returned to control level upon wash ($n=3$, $p<0.05$; Fig. 14A-C). In contrast, three minutes of DA application at 30 or $100 \mu\text{M}$ eliminated spontaneous action potentials for a period of 4.4 ± 0.5 min ($n=4$; Fig. 15A-C). Also DA at these concentrations hyperpolarized orexin neurons from a RMP of -43.1 ± 1.7 mV to -53.0 ± 2.0 mV ($n=4$; $p<0.0005$).

4.7 Bidirectional effect

It is possible that the bidirectional effect of DA results from a subpopulation of orexin neurons that responds only to D1-receptor activation whereas another population responds to D2-like receptor activation. To test whether this was the case, recordings were made during sequential application of drugs having opposite effects (agonists or different concentrations of DA; Fig. 16A-C; $n=3$). We found that every cell tested was able to respond in both directions: D1-like receptor activation increased mEPSCs or action potential frequency, whereas D2-like receptor activation induced a reduction in mEPSCs or action potential frequency. We conclude that, both D1- and D2-like receptors can modulate excitatory synaptic transmission in a single orexin neuron.

4.8 Dopamine effect on MCH neurons

In current clamp mode, $100 \mu\text{M}$ DA was found to hyperpolarize the MCH

neurons (cell 1 from -60.9 mV to -72.6 mV and Cell 2 from -62.9 mV to -68.1 mV)(n=2; Fig. 17A), suggesting that DA may also plays a significant role in modulating the physiological function of MCH neurons. As described in Section 4.1, MCH neurons were quiet at rest in our recording condition, but positive current injection caused these cells to fire action potentials (Fig. 17B, left panel). In the presence of DA, the input resistance of the cell decreased and the same positive current either failed to induce firing or induced a smaller number of spikes (Fig. 17B, center panel). This effect was reversible upon washout of DA (Fig. 17B, right panel).

To determine whether modulation of excitatory synaptic transmission underlined the inhibitory effect of DA, we switched to voltage clamp mode and DA effect was tested on mEPSCs (Fig. 18). 1 μ M DA had no effect on mEPSC frequency or amplitude (n=3). During the application of 10 μ M DA, it increased the frequency of mEPSCs in three of five cells while the remaining two neurons did not reveal a change. When 100 μ M DA was applied, one of four neurons increased the frequency of mEPSCs, one did not change and two showed a decrease in mEPSCs frequency. Overall, none of the concentrations tested (1, 10, and 100 μ M) had a significant effect ($p>0.05$) on mEPSCs in MCH neurons. Therefore, EPSC modulation does not underline DA's effect on MCH neurons. These results suggest that the action of DA on MCH neurons may be distinct from that of orexin neurons.

5 Discussion

5.1 Neuronal phenotype

Our first step was to characterize orexin and MCH neurons according to their electrophysiological features, in order to facilitate our ability to distinguish the type of LH/PFA neurons under investigation. Our results reveal that approximately 97% of the neurons recorded and selected according to their size (10-20 μm in diameter) and their proximity to the fornix were either orexin or MCH neurons.

The electrophysiological characteristics of individual neurons were investigated using a current clamp protocol that applies a series of hyperpolarizing and depolarizing steps. MCH neurons were easily identifiable by their distinct pattern of responses categorized as Type 2, including lack of I_h current, strong spike adaptation and relatively hyperpolarized RMP. Orexin neurons also had distinct features including I_h current, depolarized RMP and spike adaptation, and composed a majority of Type 1 neurons. These characteristics are similar to those described in previous studies (Burdakov et al., 2004; Eggermann et al., 2003; Gao et al., 2003; Jo et al., 2005).

In our preparation, orexin neurons were more frequent than MCH neurons (82.5% vs. 14.3% among 63 neurons analyzed). The difference in the distribution may be influenced by the dissection technique, which affects the survival of MCH neurons in our preparation. An other possibility is that the anatomical distribution of MCH neurons is different in relationship with the fornix (Baldo et al., 2004).

We also observed seven neurons that were neither orexin nor MCH immunoreactive. Some had distinct electrophysiological features and were categorized as an independent group (Type 3). Other neurons were similar to orexin neurons (Type 1). Non-orexin, non-MCH neurons we have observed in this study may correspond to other neuronal types that are known to exist in the LH/PFA. Expression of different neurotransmitters/modulators has been observed, including galanin (Melandar et al., 1986; Wortley et al., 2003; Skofitsch and Jacobowitz, 1985), neurotensin (Allen and Cechetto, 1995; Elias et al., 2000), calcitonin gene-related peptide (CGRP) (Skofitsch and Jacobowitz, 1985) and acetylcholine (Tago et al., 1987) has been observed in the LH/PFA. There is a possibility that some of these neurons did not reveal peptide immunoreactivity as a result of technical problems. For example, if the penetration of the antibody in the tissue is poor and the recorded cell was located deep in the section, the neuron may not stain. However, we excluded this possibility since we were able to detect orexin-A and MCH expression in neighboring neurons in the same layer where the biocytin-filled cell was located that failed to express orexin-A or MCH.

Our results demonstrated that distinct electrophysiology characteristics of orexin and MCH neurons can be used as reliable criteria to identify neurons during recording.

5.2 Dopamine effect on orexin neurons

The present study demonstrates that DA modulates excitatory synaptic transmission in a concentration-dependent and reversible manner in orexin neurons. The

direction of modulation depends on distinct types of receptors. D1-like receptors mediate the effect of low concentration DA (1 μ M) that induce facilitation whereas D2-like receptors mediate the effect of higher concentration (10-100 μ M) that diminish the frequency of spontaneous excitatory transmission. These changes occurred in the absence of any alteration in the amplitude of mEPSCs, from which we conclude that DA affects the transmitter release probably at the presynaptic terminal, but does not change the postsynaptic sensitivity. Furthermore, low and high concentration of DA results in an increase and decrease in action potential firing of orexin neurons, respectively, suggesting that DA modulation of spontaneous excitatory synaptic transmission may translate into altered postsynaptic firing. Indeed, it has been shown that enhancing spontaneous excitatory transmission increases the firing rate of orexin neurons (Li et al., 2002).

The hyperpolarizing effect of DA on orexin neurons is in agreement with the previous reports that employed high DA concentration (30-300 μ M) (Li and van Den Pol, 2005; Yamanaka et al., 2006), resulting in cessation of firing (Li and van Den Pol, 2005) and a reduction in intracellular calcium (Tsujino et al., 2005). Inhibition of TTX-insensitive spontaneous excitatory synaptic current shown in the present study may at least partially account for the reduced activity of orexin neurons. A direct postsynaptic effect causing a sustained outward current (Yamanaka et al., 2006) is also a potential mechanism, although we did not observe a sustained current in the presence of DA in our preparation. This discrepancy may reflect the lower concentration used in our study [100 μ M vs. 300 μ M; (Yamanaka et al., 2006)] and hyperpolarized holding potential (-80 vs.

-60 mV).

5.3 Bidirectional effect

The concentration-dependent bidirectional effect of DA is somewhat similar to that observed in the inhibitory transmission and NMDA receptor modulation in the prefrontal cortex (PFC). In the PFC slice, DA has been shown to induce a biphasic effect on the amplitude of evoked IPSCs: D2-like mediated inhibition was followed by a long-lasting D1-like receptor-mediated increase, with the D1-mediated effect apparent at a lower concentration (Seamans et al., 2001; Trantham-Davidson et al., 2004). Zheng et al. (1999) demonstrated that NMDA receptor currents were similarly modulated, with low concentration of DA inducing enhancement and high concentration inducing inhibition (Zheng et al., 1999).

Nonetheless, the mechanisms underlying the dopaminergic modulation in the prefrontal cortex (PFC) and orexin neuron are likely different. In the PFC, presynaptic D1 receptors cause a long-lasting increase in GABA release, and postsynaptic D2 receptors modulate the phosphorylation state of postsynaptic GABA receptors (Trantham-Davidson et al., 2004). Also, DA can modulate postsynaptic glutamatergic receptors such as synaptic expression of AMPA receptor (Sun et al., 2005; Zou et al., 2005) or NMDA receptor function (Zheng et al., 1999). In orexin neurons, we did not observe any change in the amplitude of mEPSCs indicating no effect on synaptic glutamatergic receptors. In addition, the effect of the D1 receptor was readily reversible

after washout of the ligand.

The mechanism for differential concentration dependency of D1 and D2 receptor-mediated modulation is unknown. It may involve different affinity state of the receptors (Seeman and Van Tol, 1993) and/or differential expression of D2 receptor isoforms that have distinct impact on excitatory transmission (Centonze et al., 2004). Interestingly, when 1 μ M DA was tested in the presence of the D1-like receptor antagonist SCH23390, an inhibitory effect was uncovered. It is possible that at 1 μ M, DA has some effect on D2-like receptors in addition to activating D1-like receptors. May be this inhibitory effect of D2-like receptors was masked by the effect of DA on D1-like receptors. In contrast, the effect of 100 μ M DA was blocked by the D2-like receptor antagonist sulpiride, and there was no underlying excitatory effect of DA.

We also observed that the opposite effects of DA could be observed in the same postsynaptic neuron. Thus we conclude that D1- and D2-like receptors can induce the opposite effects on synapses converging onto the same neuron. In fact, it is possible that D1- and D2-like receptors exert opposing effects on the same signaling pathway in the presynaptic terminal. D1 receptor activation is known to positively affect adenylyl cyclase, whereas D2 receptor activation negatively affects it (Missale et al., 1998). Activation of adenylyl cyclase and subsequent activation of cAMP and protein kinase A signaling may facilitate spontaneous neurotransmitter release, as shown in number of other synapses including those in the hypothalamus (Chavez-Noriega and Stevens, 1994; Chen and Regehr, 1997; Hirasawa and Pittman, 2003).

5.4 Dopamine effect on MCH neurons

We observed that in MCH neurons, DA induces a hyperpolarization that accompanies a reduction in the rate of action potential firing in response to positive current injections. Based on this preliminary observation, an ongoing project in our laboratory has discovered that DA induces a direct, sustained outward current in MCH neurons in a concentration-dependent manner. Surprisingly, this effect is mediated by alpha 2-adrenoreceptors, not by DA receptors (Trask and Hirasawa, 2005). This finding is in agreement with a previous publication where norepinephrine reduces spike frequency and hyperpolarized MCH neurons through alpha 2-adrenoreceptor activation (van Den Pol et al., 2004).

The present study also suggests that DA has some effect on the excitatory synaptic inputs to MCH neurons. The magnitude and the direction of effects varied among cells tested and the concentration of DA used but bidirectional effects were never observed in a given neuron. We propose that there are subpopulations of MCH neurons that respond differently to DA. The precise cellular mechanism by which DA modulates the activity of MCH neurons remains unknown and needs more studies.

5.5 Physiological implication

There is a reciprocal communication between the mesolimbic DA system and the LH/PFA, more specifically orexin and MCH neurons. Orexin neurons are known to send direct projections to the ventral tegmental area (VTA) (Fadel and Deutch, 2002) and

exhibit excitatory effect there (Borgland et al., 2006; Harris et al., 2005; Korotkova et al., 2003a; Vittoz and Berridge, 2006), which leads to DA release in nucleus accumbens (NAcc) (Narita et al., 2006) and prefrontal cortex (PFC) (Vittoz and Berridge, 2006). MCH containing fibers are also found within the VTA (Dallvechia-Adams et al., 2002), although MCH has no apparent effect on the activity of VTA neurons (Korotkova et al., 2003b). In contrast, MCH is known to inhibit DA-induced AMPA receptor phosphorylation in medial spiny neurons of the NAcc (Georgescu et al., 2005). This effect would reduce the AMPA receptor-mediated current in these neurons. Therefore, MCH also modulates the functioning of the mesolimbic DA system. This mechanism at least partially accounts for the excitatory effect of MCH on food intake (Georgescu et al., 2005).

On the other hand, the VTA supplies a bulk of the DA input to the LH/PFA (Leibowitz and Brown, 1980; Yoshida et al., 2006). DA in the LH/PFA reduces food intake, reward response and locomotion (Leibowitz, 1975; Leibowitz and Rossakis, 1978; Parada et al., 1988; Yang et al., 1997). Our study provides a cellular mechanism by which DA suppresses the activity of LH/PFA neurons. We propose that DA directly inhibits MCH neurons. Also we have demonstrated that DA inhibits orexin neurons at higher concentrations through synaptic inhibition. Given the orexigenic effects of MCH and orexins, the anorexic action of DA in the LH/PFA may be due to inhibition of MCH and orexin neurons.

Based on the current study, in combination with previous reports, we propose a concentration-dependent dual feedback mechanism between the orexin and mesolimbic

DA system (Fig. 19). A low/moderate level of DA in the LH/PFA may excite orexin neurons through D1-like receptor-mediated facilitation of excitatory input, which in turn provides excitatory influence on VTA neurons, thus creating a positive feedback mechanism. When dopaminergic activity is elevated and a higher concentration of DA is achieved in the hypothalamus, this will work to activate D2-like receptors and inhibit orexin neurons. This will lead to decreased excitatory input to the VTA, acting as a negative feedback mechanism. Such two-way communication between the LH/PFA and the mesolimbic DA pathway is likely to be critical for optimal control of appetite, reward value of food and feeding.

6 Conclusion

Obesity is a serious global health problem of epidemic proportions around the world (DeNoon, 2007; World Health Organization, 2007). Canadian society is not an exception where the overconsumption of high-energy food contributes to the rising problem of obesity, which is in turn a high risk factor for various chronic diseases (World Health Organization, 2007). In order to understand the etiology of obesity, it is important to comprehend how energy homeostasis and motivation to eat food is controlled. Since 1962, the LH has been recognized as a brain area where feeding and reward are regulated (MARGULES and OLDS, 1962) and orexin and MCH neurons are found in this area (Nahon, 1994; Sakurai et al., 1998). DA releases in the LH/PFA is known to modulate feeding reward (Fetissov et al., 2000; Fetissov et al., 2002; Sato et al., 2001). Therefore, we hypothesized that DA modulated orexin and MCH neurons. To test this hypothesis, this thesis focused on the mechanism of DA effect on orexin and MCH neurons at the cellular level, which play an important function in reward and energy homeostasis.

We demonstrated a dose-dependent bidirectional effect on the activity of orexin neurons via modulation of excitatory transmission at the presynaptic terminals. In contrast, DA inhibits MCH neurons in a dose dependent manner without a significant effect on excitatory transmission. Thus, the results of my theses project suggest that DA has an effect on MCH neurons through a mechanism different from that of orexin neurons. Nonetheless, inhibitory effect of DA on both types of neurons provides a negative feedback mechanism to prevent overactivation of the reward circuit.

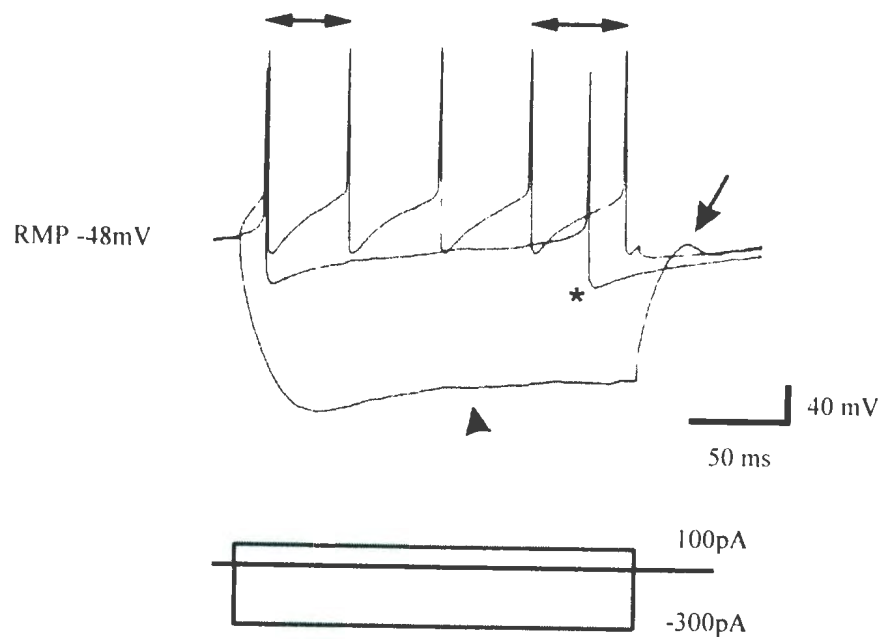
Overall, these data suggest that DA plays an important role in the modulation of the LH/PFA function, vital players in the control of food intake, energy expenditure and food reinforcement. Perhaps, disruption of the physiological DA function within the LH/PFA may result in the excessive food intake and disruption of metabolism leading to obesity.

Figure 1: Electrophysiological characteristics of Type 1 neuron

A: In fifty-two neurons, hyperpolarization induced a sag characteristic of an Ih current (arrow head) and rebound depolarization at the current offset (arrow). Also spontaneous firing (*) and no adaptation (\leftrightarrow) are shown. Lower panel indicates current clamp protocol of a series of 200 ms-step pulses.

B: A representative cell showing characteristic of Type I with a resting membrane potential (RMP) lower than -60mV. Lower RMP corresponds to a non-orexin, non-MCH neuron. Lower panel indicates current clamp protocol of a series of 200 ms-step pulses.

A



B

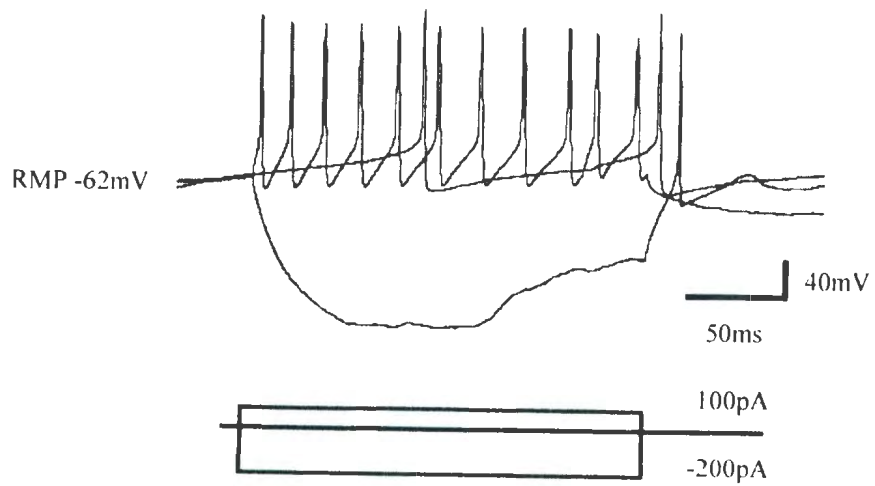


Figure 1

Figure 2: Electrophysiological characteristic of Type 2 neuron

Nine neurons displayed neither I_h current nor rebound depolarization, but showed a strong spike adaptation. Lower panel is indicating a series of 200 ms-step pulses.

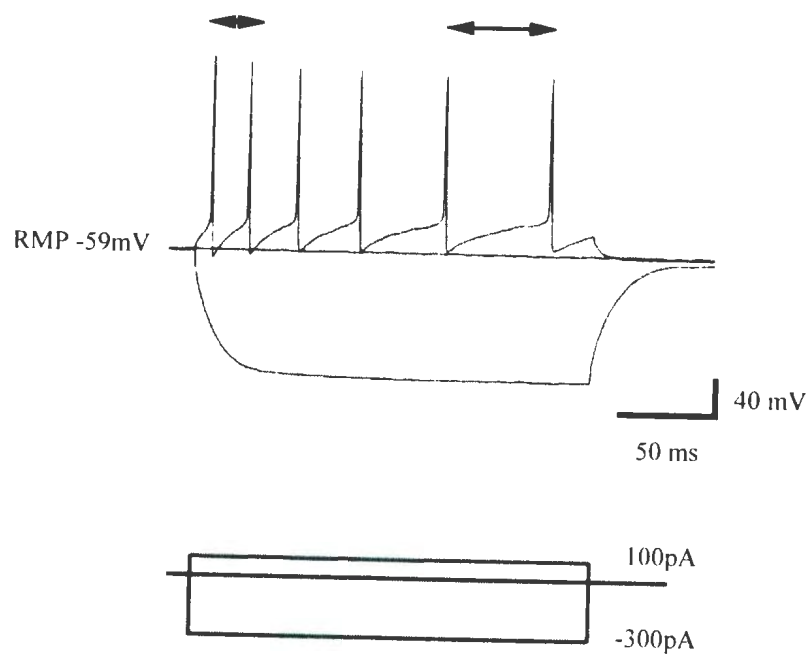


Figure 2

Figure 3: Electrophysiological characteristics of Type 3 neuron

Two neurons displayed no I_h current, but fired an action potential following a hyperpolarizing current step. A positive current injection induced only one action potential which then ceased all activities. Lower panel is indicating a series of 200 ms-step pulses.

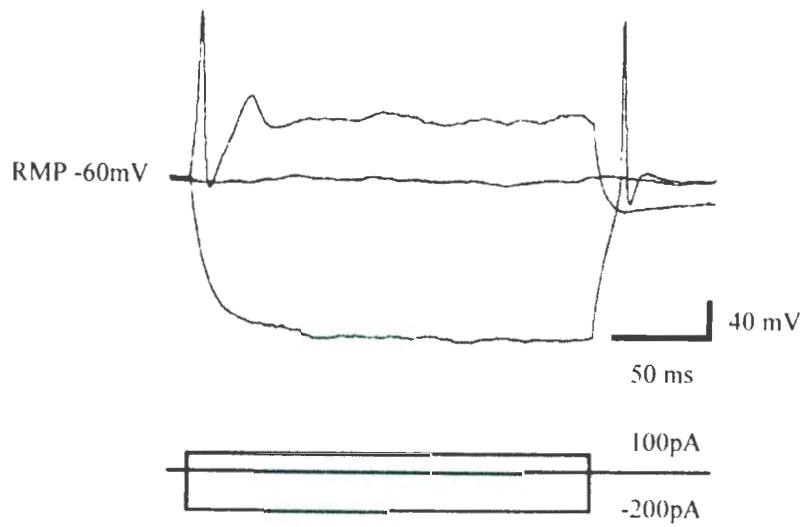


Figure 3

Figure 4: Immunohistochemical characterization of recorded neurons

A: (Left panel) An example of a cell filled with biocytin during recording.

(Middle panel) Orexin A immunoreactivity shown in red.

(Right panel) Overlay showing the biocytin labeled cell is orexin A immunopositive.

B: (Left panel) An example of a cell filled with biocytin during recording.

(Middle panel) MCH immunoreactivity shown in green.

(Right panel) Overlay showing the biocytin labeled cell is MCH immunopositive.

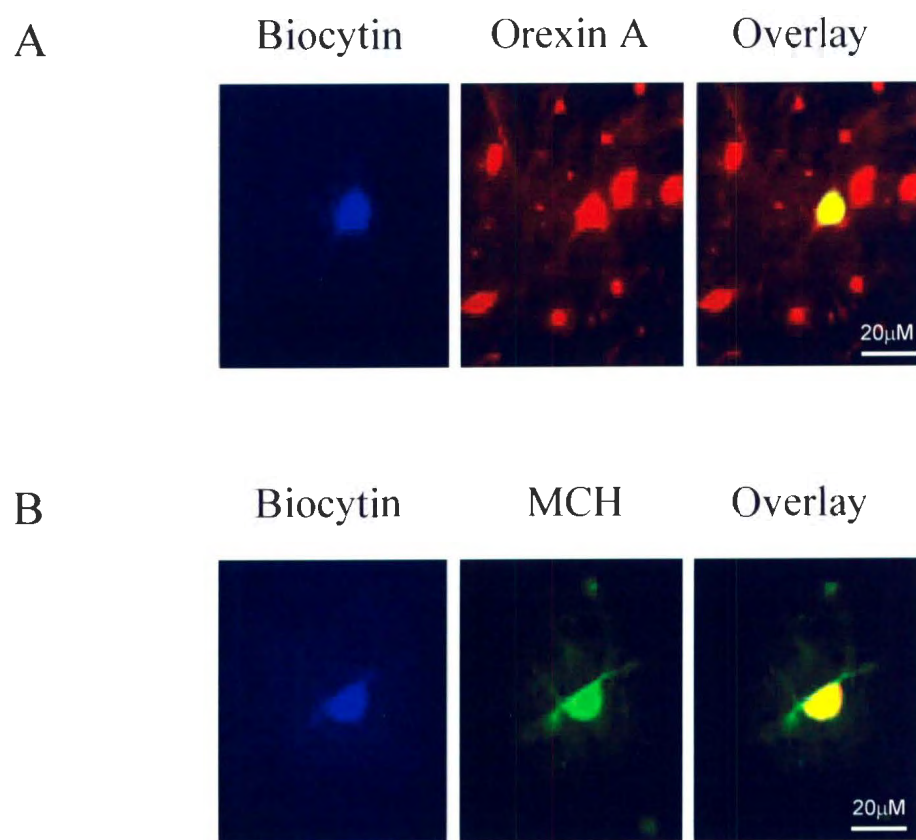


Figure 4

Figure 5: Phenotyping of PFA/LH neurons

A diagram showing different cells types in the PFA/LH divided by their electrophysiological characteristics and confirmed by immunohistochemical technique.

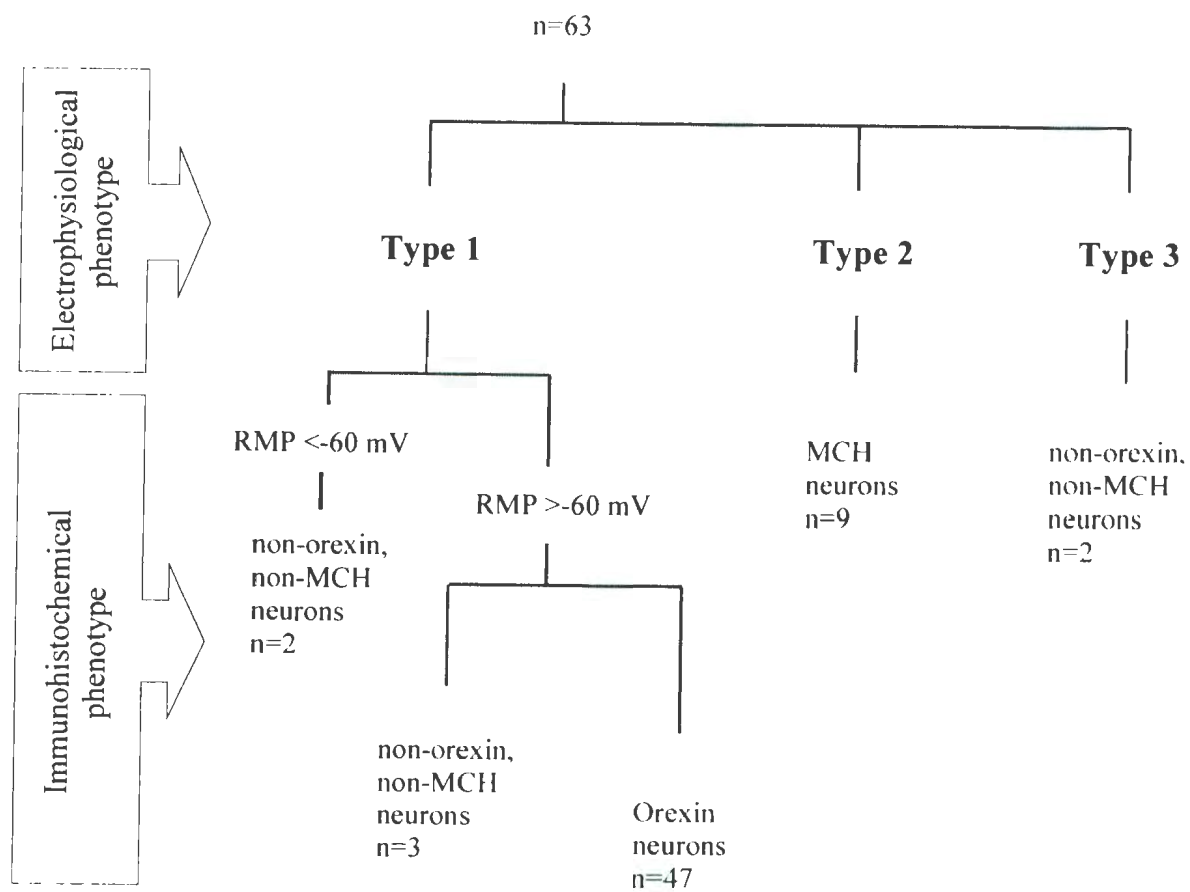


Figure 5

Figure 6: Spontaneous synaptic currents in orexin neurons

(left panel) Representative traces from an orexin neuron showing miniature EPSCs recorded in the presence of picrotoxin and TTX.

(right panel) Almost all events were abolished by addition of DNQX.

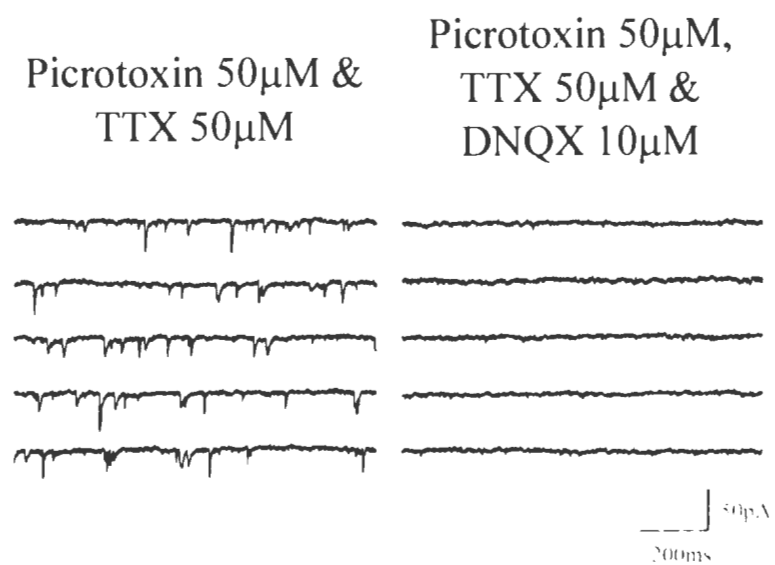


Figure 6

Figure 7: Low concentration of dopamine increases excitatory transmission

A: Sample traces showing mEPSCs in control condition, in the presence of dopamine (DA) ($1\mu\text{M}$) and after wash as indicated.

B: A time-effect plot of the frequency of mEPSCs in a representative cell in presence of DA ($1\mu\text{M}$).

C: Cumulative plot of inter event interval.

D: Cumulative plot of amplitude of mEPSCs from the same cell as **A**.

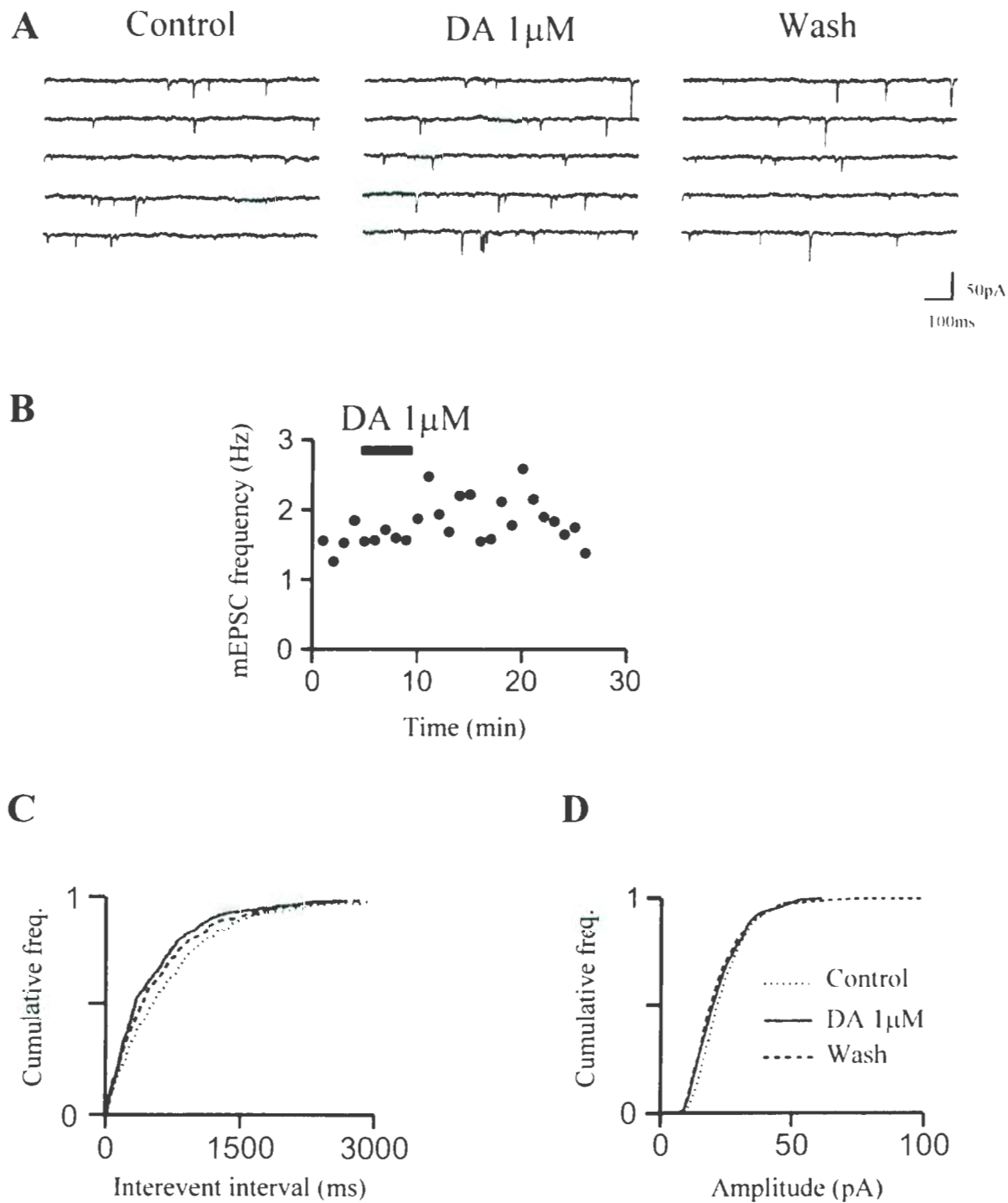


Figure 7

Figure 8: High concentration of dopamine decreases excitatory transmission

A: Sample traces showing mEPSCs in control condition, in the presence of DA (100 μ M) and after wash as indicated.

B: A time-effect plot of the frequency of mEPSCs in a representative cell in presence of DA (100 μ M).

C: Cumulative plot of interevent interval.

D: Cumulative plot of amplitude of mEPSCs from the same cell as **A**.

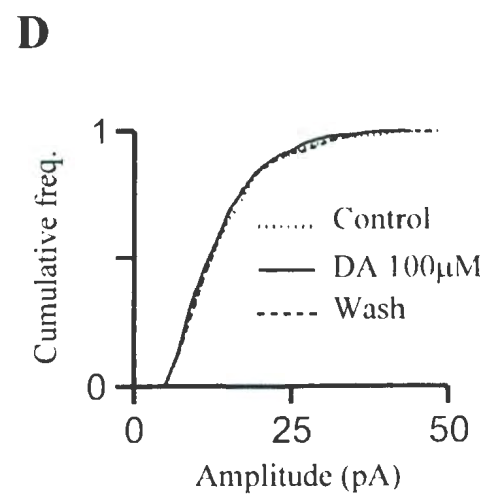
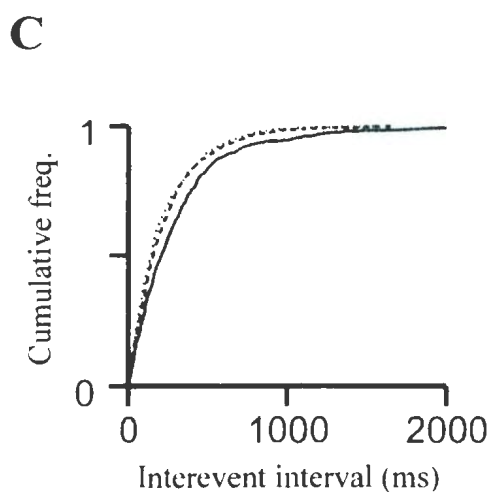
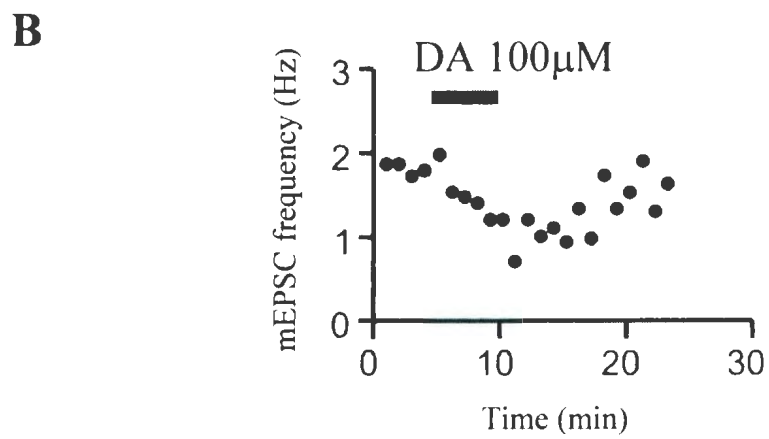
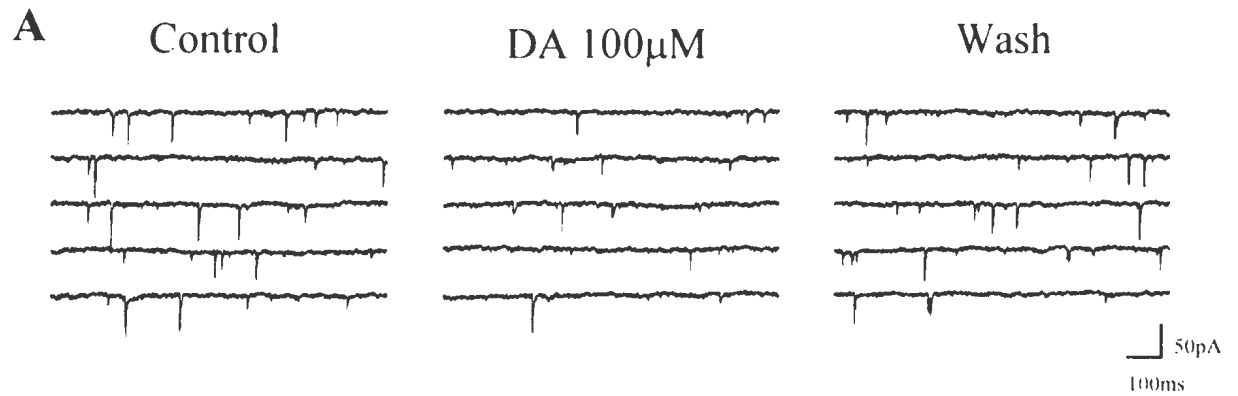


Figure 8

Figure 9: Dopamine induces a reversible bidirectional changes in spontaneous excitatory transmission

Bar graph showing a summary of the effect of DA on mEPSC frequency at different concentrations. $P < 0.05$: *, $P < 0.005$: ***, *t-test*.

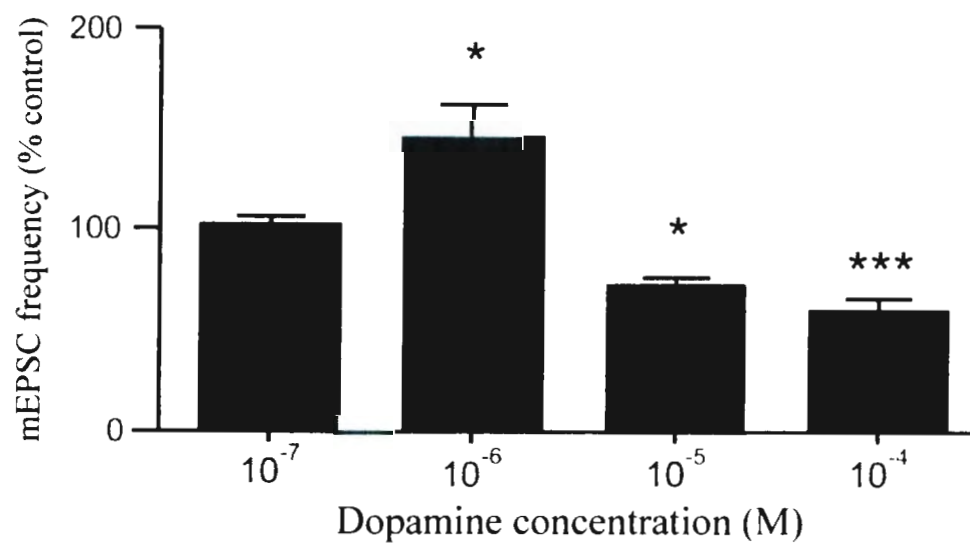


Figure 9

Figure 10: **D1-like receptor activation increases the frequency of mEPSCs**

A: Sample traces showing mEPSCs in basal condition (control), in the presence of SKF81297 (10 μ M) and after wash as indicated.

B: A time-effect plot of a representative cell showing the frequency of mEPSCs in the presence of SKF81297 (10 μ M).

C: Cumulative plot of interevent interval.

D: Cumulative plot of the amplitude of mEPSCs from the same cell as **A**.

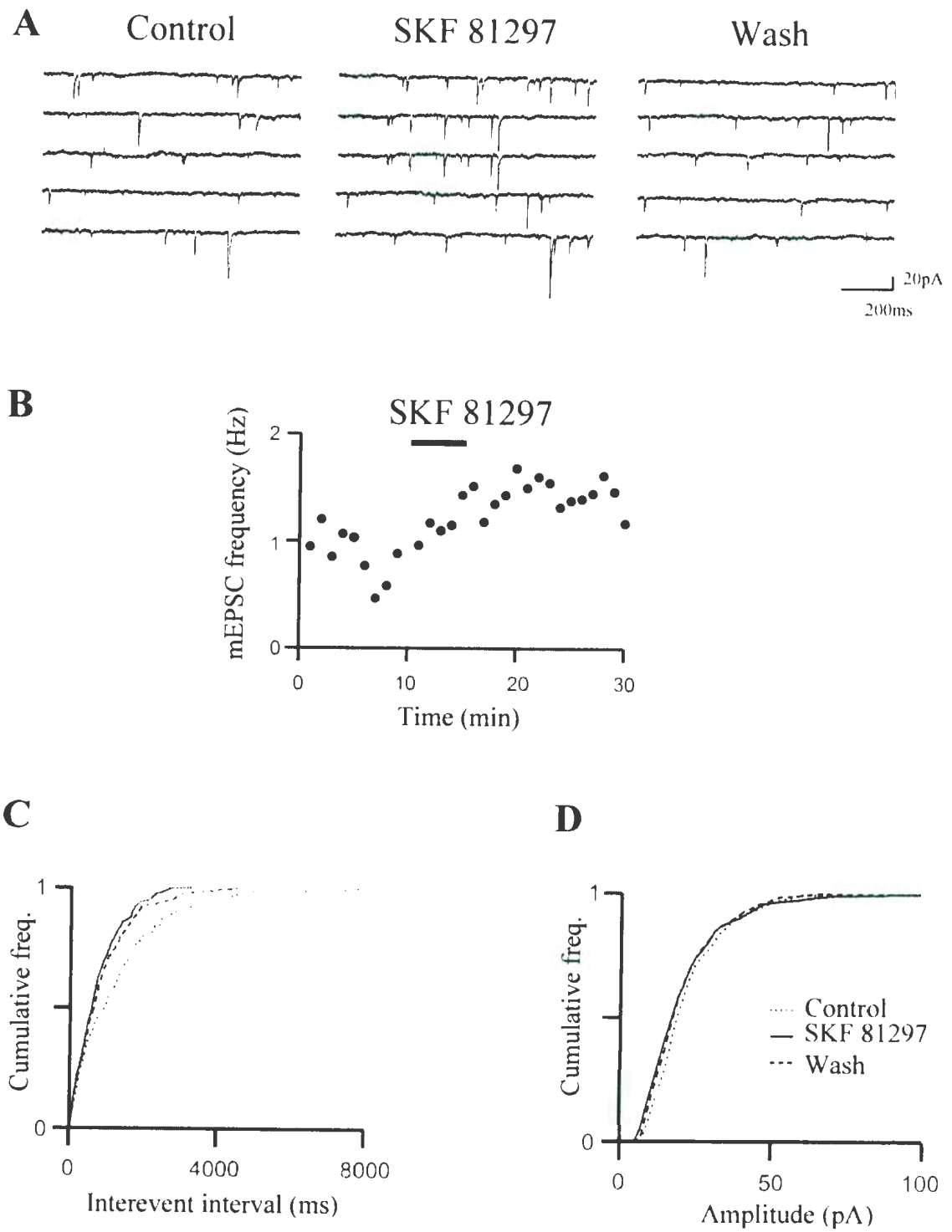


Figure 10

Figure 11: D1-like receptor activation

A: Time-effect plot of the frequency of mEPSCs depicting the response to DA (1 μ M) in a cell pre-treated with SCH23390 (10 μ M).

B: Summary of the effect of DA 1 μ M alone, DA 1 μ M in the presence of SCH23390 and SKF81297 alone on mEPSC frequency. $P < 0.05$:* compared to baseline condition, $P < 0.01$:**. n. s.: no significant, *t-test*.

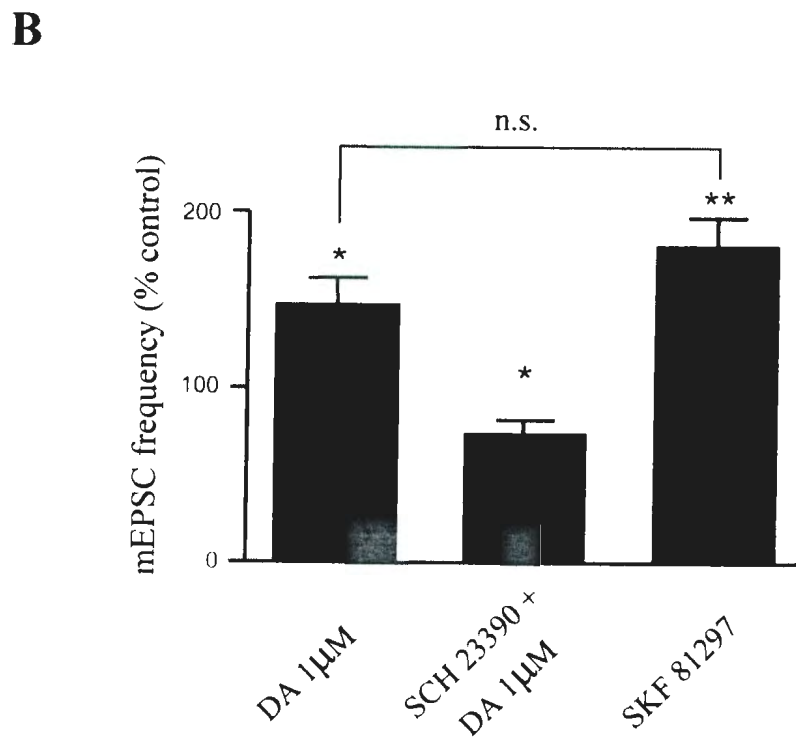
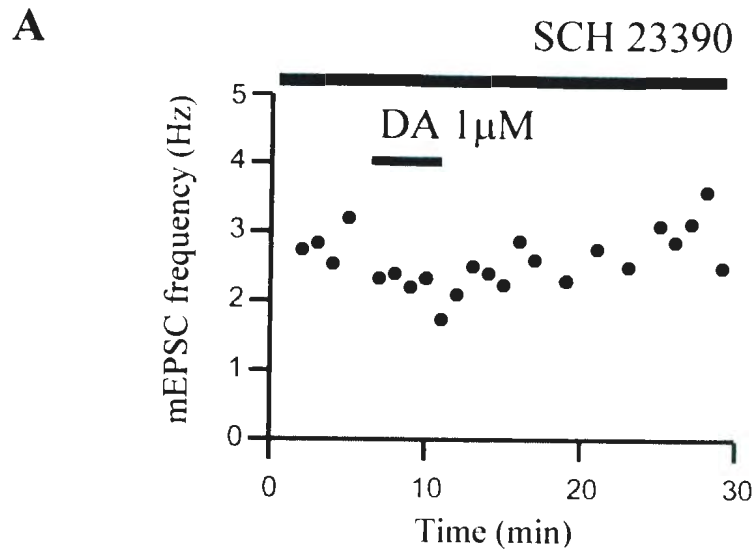


Figure 11

Figure 12: **D2-like receptor activation decreases the frequency of mEPSCs**

A: Sample traces showing the basal mEPSCs (control), in the presence of quinpirole (10 μ M) and after wash as indicated.

B: A time-effect plot of a representative cell showing the frequency of mEPSC's in the presence of quinpirole (10 μ M).

C: Cumulative plot of interevent interval.

D: cumulative plot of amplitude of mEPSCs from the same cell as **A**.

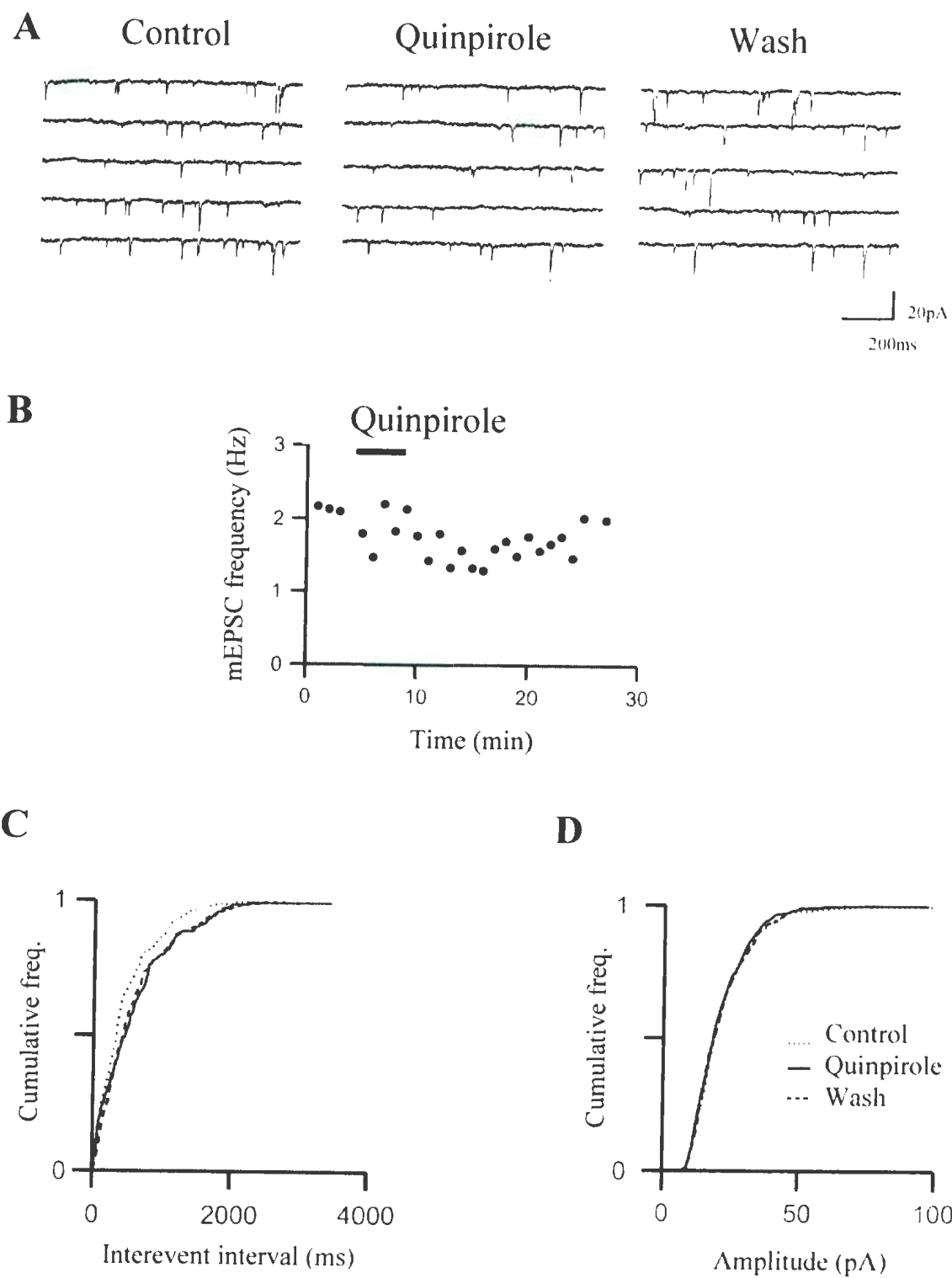


Figure 12

Figure 13: D2-like receptor activation

A: Time-effect plot of the frequency of mEPSCs depicting the response to DA (100 μ M) in a cell pre-treated with sulpiride (10 μ M).

B: Summary of the effect of DA 100 μ M, Sulpiride plus DA 100 μ M and quinpirole (10-50 μ M) on mEPSC frequency. $P < 0.05$:* compared to the baseline condition, $P < 0.005$:***. n.s.: no significant differences between the two drug concentrations, *t-test*.

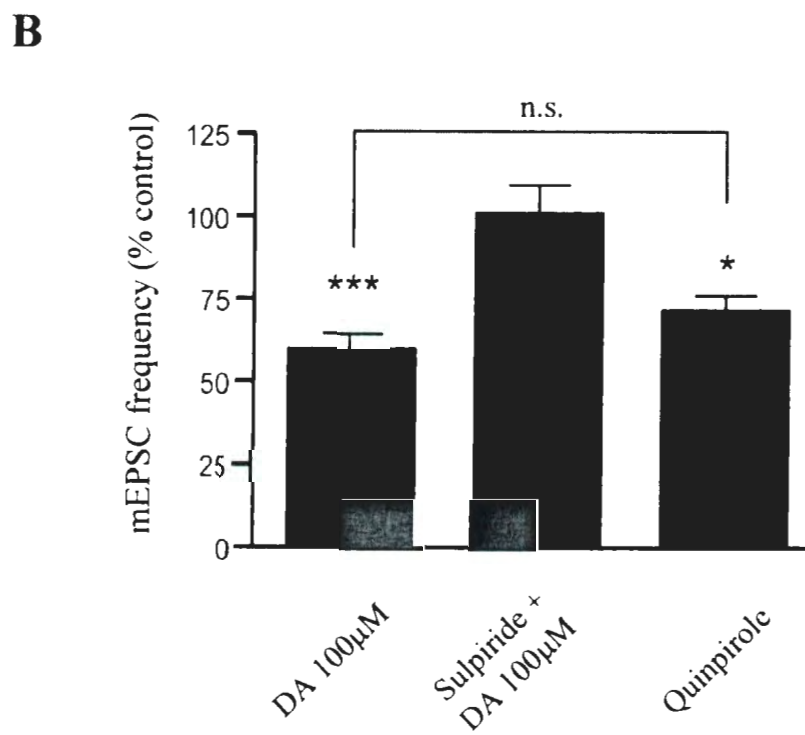
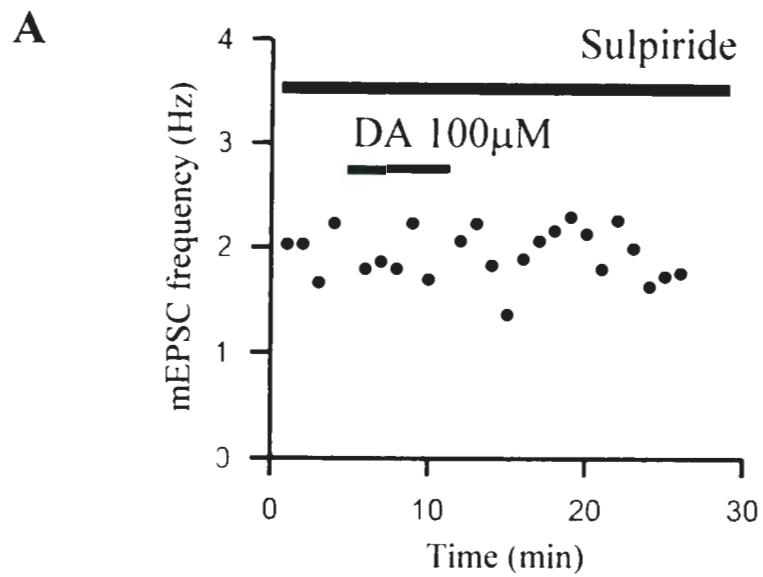


Figure 13

Figure 14: Low concentration of dopamine increases the rate of action potential in orexin neurons

A: Sample recording showing the effect of 1 μ M DA on the firing activity.

B: Expanded traces from another cell recorded before, during and after DA 1 μ M application.

C: Time-effect plot of the same experiment as shown in **B**.

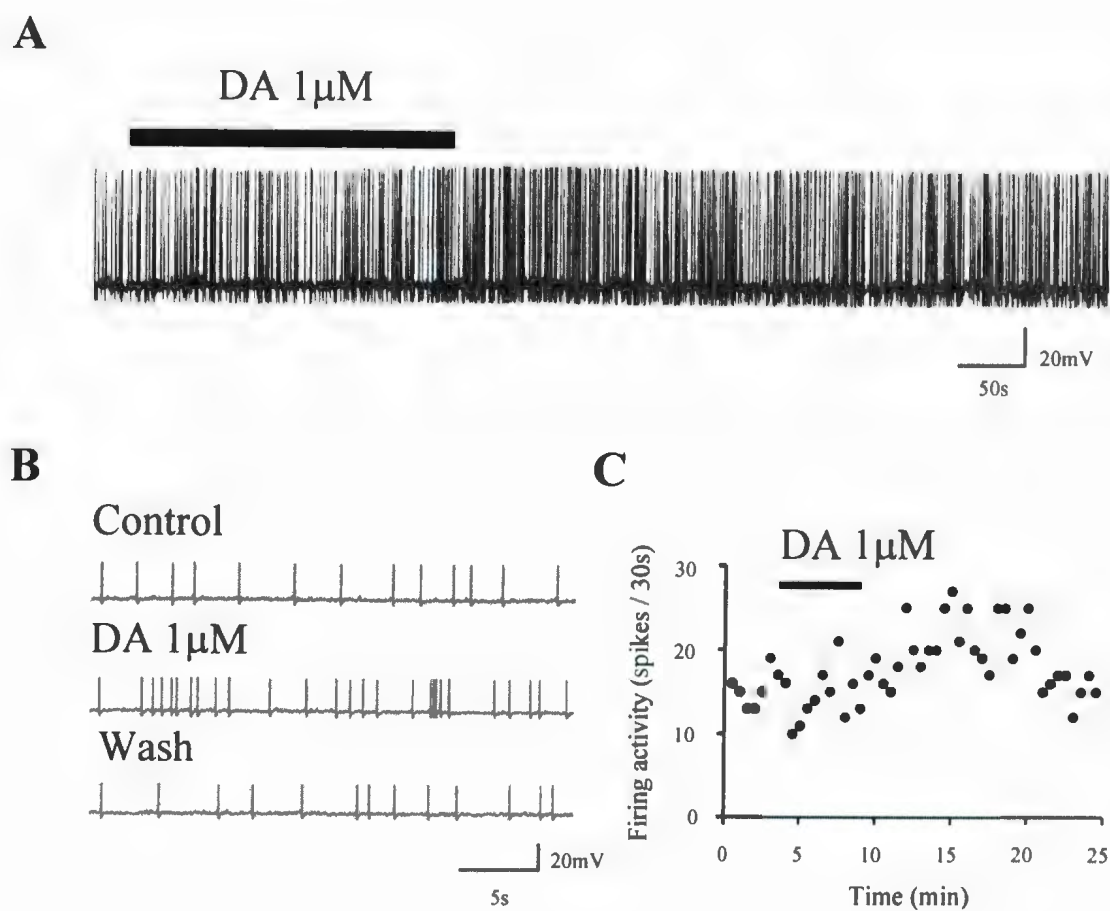


Figure 14

Figure 15: **High concentration of dopamine hyperpolarizes and diminishes the firing activity of orexin neurons**

A: Typical recording depicting the effect of 100 μ M DA on firing activity.

B: expanded traces from an other orexin neuron recorded before, during and after DA 100 μ M application.

C: Time-effect plot of the same experiment as shown in **B**.

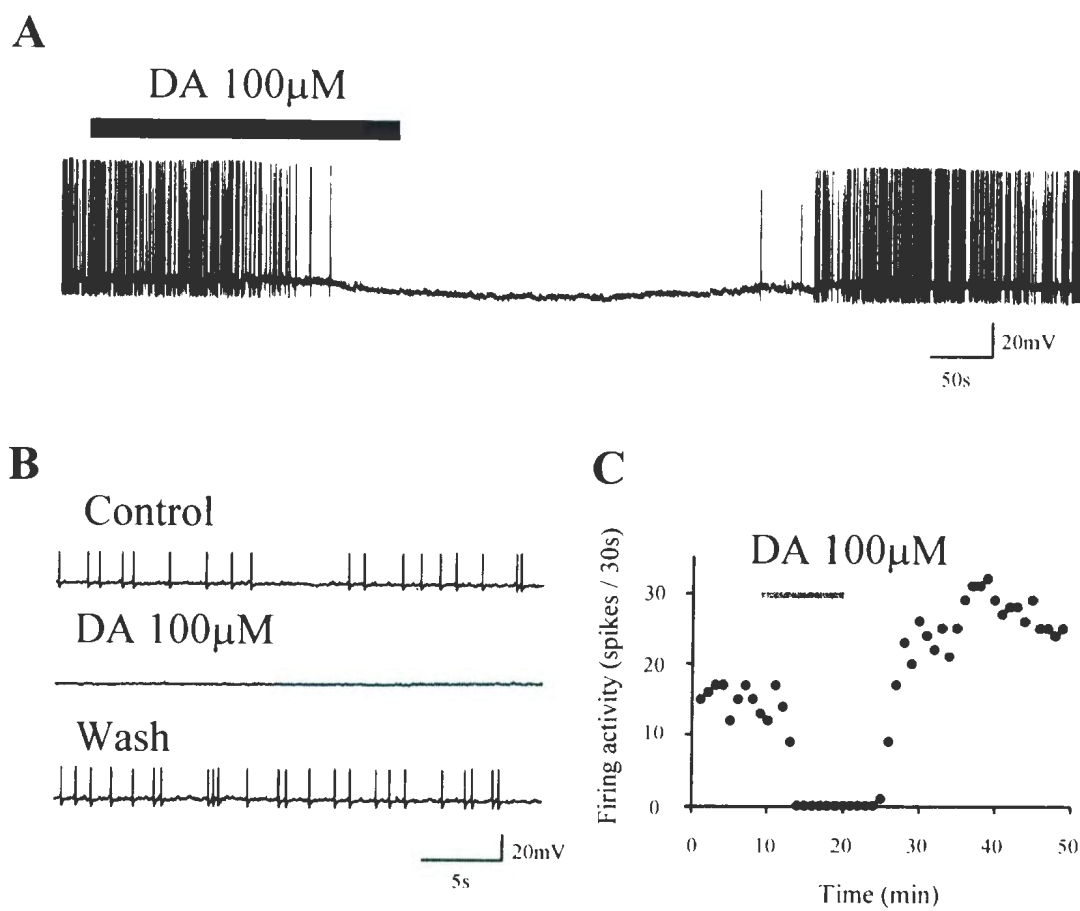


Figure 15

Figure 16: D1- and D2-like receptors modulate synaptic inputs to the same orexin neuron

A: A time-effect plot of a cell showing the frequency of mEPSCs in response to sequential applications of DA (100 μ M) and SKF 81297 (10 μ M).

B: Time-effect plot of the frequency of mEPSCs from another neuron depicting the response to consecutive application of quinpirole (10 μ M) and SKF 81297 (10 μ M).

C: A cell showing the rate of action potential firing is bidirectionally modulated by low and high dose of DA.

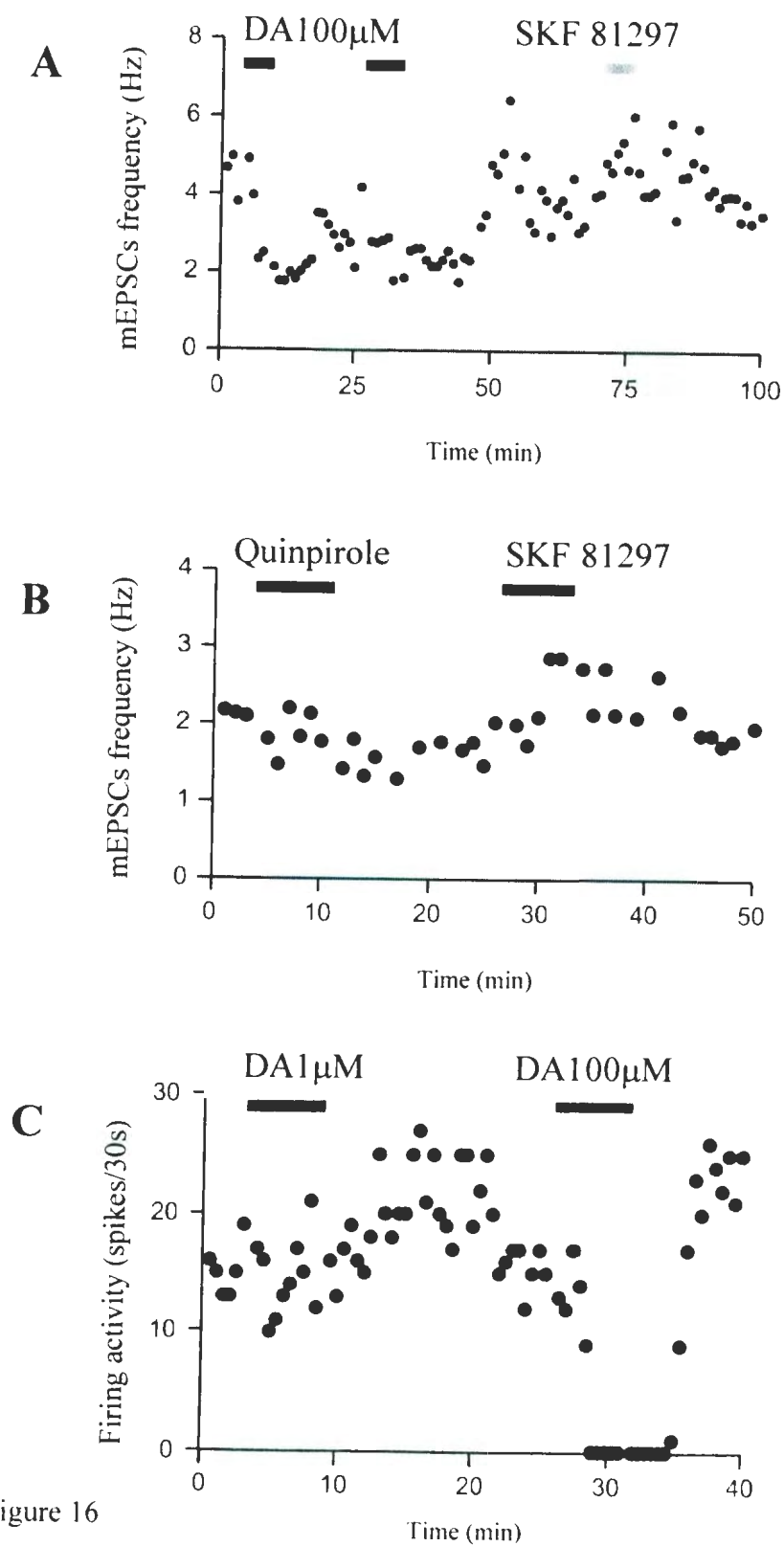


Figure 16

Figure 17: Effect of dopamine on MCH neuron

A: Sample recording in current clamp mode showing the effect of 100 μ M DA on a MCH neuron. Vertical lines depict the voltage responses to hyperpolarizing and depolarizing current step pulses applied through the recording pipette. Arrow is showing changes in the input resistance.

B: Voltage responses to positive and negative current injections recorded from the same neuron in panel A, in expanded time scale. When DA was applied the cell RMP changed from -60 to -70 mV.

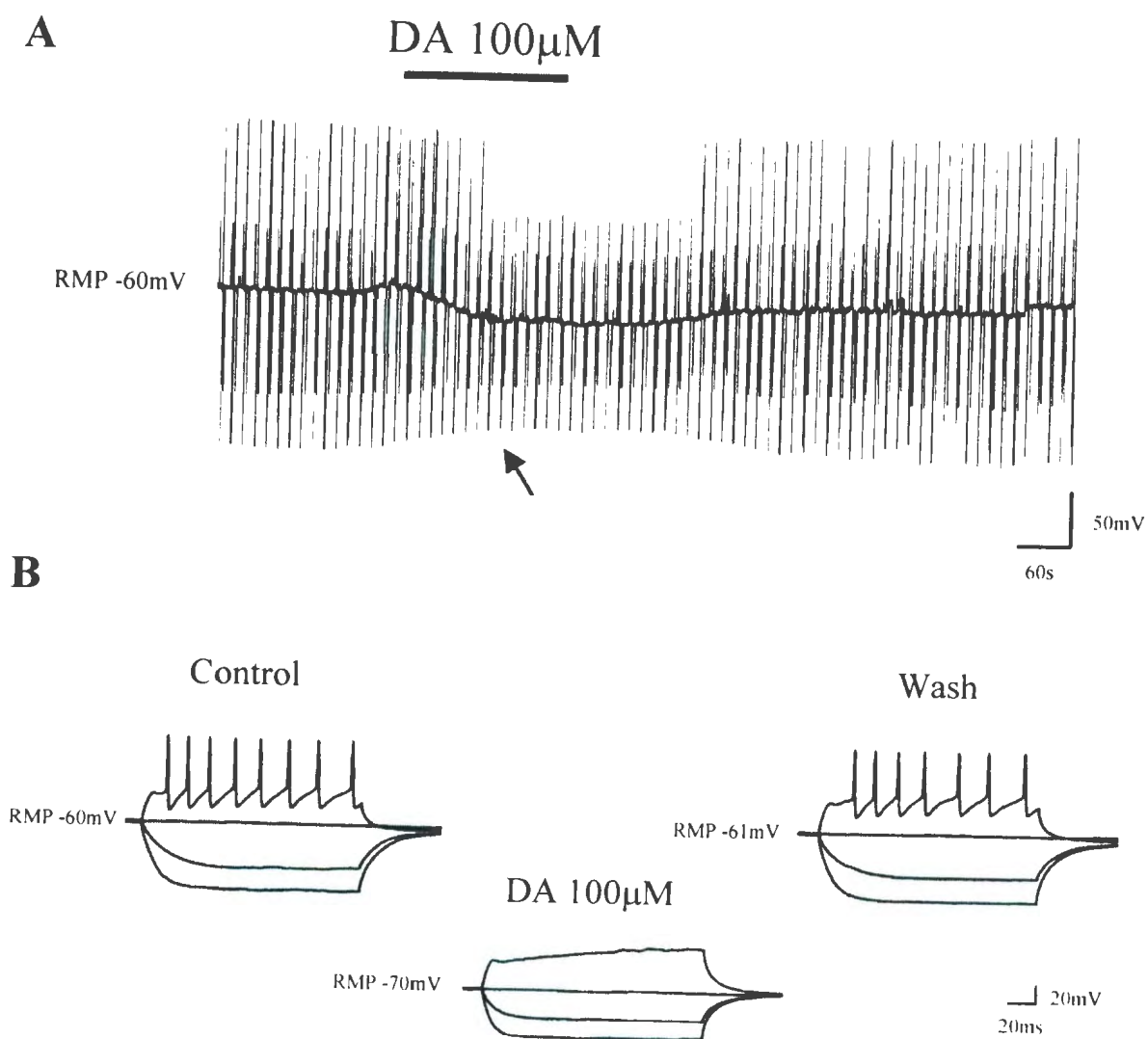


Figure 17

Figure 18: Effect of DA on mEPSCs in MCH neurons

Summary of the effect of DA 0.1, 10 and 100 μ M on mEPSC frequency in MCH neurons. Each circle denotes a cell.

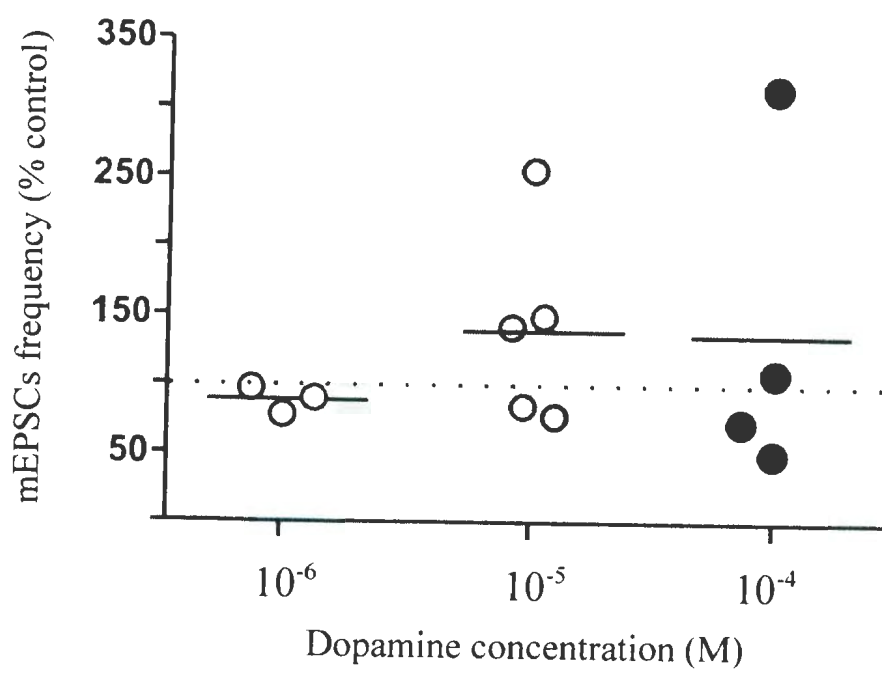


Figure 18

Figure 19: **Schematic representation of dose-dependent dual feedback**

A: Low or moderate DA input from the ventral tegmental area (VTA) to the LH/PFA (a) activates orexin neurons. Orexin neurons in turn send excitatory input from the LH/PFA to the VTA (b). This is a positive feedback loop.

B: The excitatory orexin effect in the VTA (d) increases DA release in the Nacc (e).

C: Higher orexinergic activity increases DA input from the VTA to the LH/PFA (f), which in turn inhibits orexin neurons. This acts as a negative feedback loop.

D: Less orexin input to the VTA (g) results in less DA release in the Nacc (h).

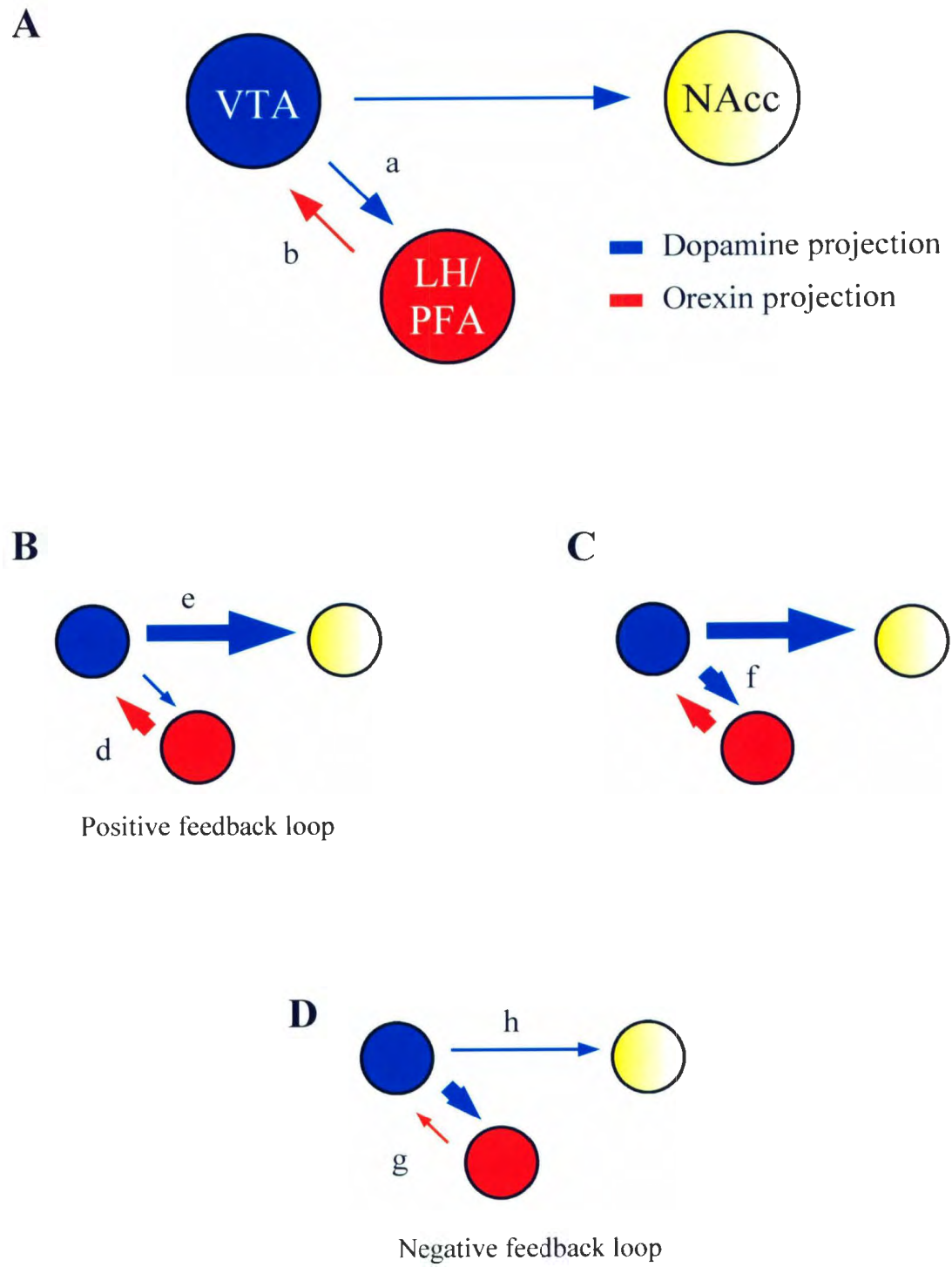


Figure 19

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